GENETIC AND PHYSIOLOGICAL STUDIES ON *RHIZOBIUM TRIFOLII*

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To KATHY

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DECLARATION

The work contained in this thesis was the result of original research conducted by myself, except for the data in Table 43 which were compiled with the aid of Dr. S.B. Primrose.

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SUMMARY

The aim of this study was to investigate the Rhizobium-legume symbiosis by use of mutants of R. trifolii. Attempts to demonstrate R68-45 mediated chromosomal recombination in R. trifolii were unsuccessful. However, the pathways and regulation of nitrogen and carbon metabolism in R. trifolii were studied.

Rhizobium trifolii assimilated ammonia solely by the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway. Neither strain W19, which lacked GOGAT, nor pyruvate-carboxylase mutants of strain 7000 could utilize glutamate-yielding amino-acids to satisfy their growth requirements. It was proposed that GS regulated the synthesis of the required catabolic enzymes, and that GS was regulated by the intracellular 2-oxoglutarate to glutamine ratio.

The Embden-Meyerhof-Parnas pathway was not found in R. trifolii. By use of mutants, it was shown that glucokinase (glk) was required for glucose phosphorylation in wild-type bacteria and the Entner-Doudoroff pathway for catabolism of all six-carbon sugars except galactose. Pyruvate carboxylase was the physiologically important anaplerotic enzyme. The effective symbiotic properties of the carbohydrate-negative mutants showed that hexoses were not the energy-substrates received by the bacteroids.

The glk mutants did not grow on glucose, maltose, cellobiose, trehalose, sucrose, lactose or dulcitol. The syntheses of the lactose and dulcitol transport systems were hypersensitive to repression by a second carbon source in the glk mutants. A model was proposed to explain the pleiotropic defect.

The catabolism of glucose to at least glucose 6-phosphate was required for the regulation of those catabolic systems which were sensitive to catabolite repression by glucose. The utilization of the polyols sensitive to catabolite repression was inhibited by 2-deoxyglucose in both wild-type and glk strains. It was proposed that the regulation of catabolic enzyme synthesis in R. trifolii was manifested mainly at the level of inducer exclusion.

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A_{540}	absorbance at 540 nm
ADP	adenosine diphosphate
AICAR	5-amino-4-imidazole-carboxamide ribonucleotide
AIR	5-amino-4-imidazole ribonucleotide
AMP	adenosine monophosphate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
C	carbon
CAIR	5-amino-4-imidazole carboxylic acid ribonucleotide
cAMP	cyclic adenosine monophosphate
Cda	chromosome donor ability
dbcAMP	dibutyryl cyclic adenosine monophosphate
DEAE	diethylaminoethyl
2DG	2-deoxyglucose
DNA	deoxyribonucleic acid
DTE	dithioerythritol
ED	Entner-Doudoroff
EMP	Embden-Meyerhof-Parnas
e.o.p.	efficiency of plating
FAICAR	5-formamido-4-imidazole carboxamide ribonucleotide
FDA	fructose diphosphate aldolase
GAP	glyceraldehyde 3-phosphate
GDH	glutamate dehydrogenase
Glc	glucose
GOGAT	glutamate synthase
G6P	glucose 6-phosphate
G6PDH	glucose-6-phosphate dehydrogenase
GS	glutamine synthetase
GSYC	glucose-salts-yeast extract-casein hydrolyzate medium
h	hour
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
IAA	indoleacetic acid
ICD	isocitrate dehydrogenase
KDPG	2-keto-3-deoxy-6-phosphogluconate
Kn^R	kanamycin-resistant

legHb	leghaemoglobin
legHb.O_2	oxygenated leghaemoglobin
LPS	lipopolysaccharide
αMG	α-methylglucoside
N	nitrogen
NADH	β-nicotinamide adenine dinucleotide (reduced form)
NADPH	β-nicotinamide adenine dinucleotide phosphate (reduced form)
nmol	nanomole
NTG	N-methyl-N'-nitro-N-nitrosoguanidine
ONPG	orthonitrophenyl-β-D-galactopyranoside
PEP	phosphoenolpyruvate
PFK	phosphofructokinase
6PG	6-phosphogluconate
6PGDH	6-phosphogluconate dehydrogenase
PHB	poly-β-hydroxybutyrate
PNPG	paranitrophenyl-β-D-galactopyranoside
PP	pentose phosphate
PTS	phosphoenolpyruvate: sugar phosphotransferase system
PYR	pyruvate
RDM	defined medium
SAICAR	5-amino-4-imidazole-N-succinocarboxamide ribonucleotide
SAM	sorbitol, arabitol and mannitol
SVD	snake venom phosphodiesterase
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride
Y_{ATP}	growth yield (g) per mole of ATP
YGA	yeast extract-glucose agar medium
YM	yeast extract-mannitol medium
YMA	yeast extract-mannitol-agar medium

CHAPTER 1

LITERATURE REVIEW

1.1 General Introduction

The main interest in the genus Rhizobium lies in its ability to form a symbiotic relationship with leguminous plants which results in the fixation of dinitrogen and the supply of the reduced product to the plant. Biological nitrogen fixation contributes approximately two-thirds (175×10^6 tonnes) of the earth's annual production of fixed nitrogen, and of this amount 80×10^6 tonnes are fixed by nodulated legumes grown for agricultural purposes (Burns and Hardy, 1975). Total world consumption of nitrogen fertilizer increased eight-fold from 1954-1974 and in 1974 stood at 44×10^6 tonnes per annum, but this consumption was largely limited to the developed countries. Nitrogen supply is the major factor limiting food supply in less-developed countries. (Skinner, 1976). Production of nitrogen fertilizer is inextricably linked to the energy situation, with large amounts of energy required, both to provide H_2 (from natural gas) and for the Haber-Bosch process itself. Hence, due to the ever-increasing cost and decreasing availability of the energy sources, fertilizer nitrogen is becoming increasingly expensive. This has led to massive funding for research on biological nitrogen fixation (Skinner, 1976) and to speculation on the possibility of constructing nitrogen fixing strains of non-leguminous, agriculturally important plants (Shanmugam and Valentine, 1975). However, the naturally occurring symbiotic systems are still poorly understood, and research effort directed towards improving the efficiency of these systems seems to be the most promising approach for increasing biological nitrogen fixation in, at least, the short term (Gibson et al., 1977).

While recognizing the undoubted importance of the non-leguminous symbiotic systems (Burris, 1977), this literature review is confined largely to the Rhizobium-legume symbiosis. The high current interest in biological nitrogen fixation has resulted in an abundance of reviews,

symposia, etc. (Quispel, 1974; Burns and Hardy, 1975; Zumft and Mortenson, 1975; Newton and Nyman, 1976; Nutman, 1976; Hardy and Gibson, 1977; Hardy and Silver, 1977; Hollaender, 1977; Newton et al., 1977), and readers are referred to these volumes for supplementary details on most topics. This review covers the basic classification of rhizobia, a basic outline of nodule formation, with detailed description of post-1975 research only, the basic biochemistry and physiology of the nitrogenase system and the constraints it places on biological nitrogen fixation, some of the biochemical processes occurring in the mature nodule and their analogues in free-living rhizobia, the use of a 'genetic' approach to delineate 'who does what' in the nodule, genetic exchange in rhizobia, and the aims of this study.

1.2 Rhizobium taxonomy

The Rhizobium-legume symbiosis is the result of a complex interaction involving differentiation of both partners. An important feature of this interaction is its specificity. In general, rhizobia which nodulate, for example, clover will not nodulate soybean, peas, etc., and similarly those that nodulate soybeans will not nodulate clover, peas, etc. This 'host specificity' has provided the basis for the definition of the genus Rhizobium into six species (Table 1). However, this taxonomy ignores the large number of rhizobial strains that nodulate other legumes, e.g. lotus rhizobia, cowpea rhizobia, and also the large variability found within a single species of Rhizobium in the ability to nodulate various species and indeed cultivars of the host genus. It is thus highly unsatisfactory, except perhaps for agronomic purposes (Graham, 1976). Current thought suggests that the rhizobia should be divided into two generic groups on classical taxonomic grounds. Group I would contain the 'fast-growing' rhizobial strains, which nodulate peas, beans, clover or lotus, while Group II would comprise the slow-growing rhizobial strains which nodulate soybeans, cowpeas, lupins or lotus. Rhizobium meliloti may merit a separate genus (Graham, 1976).

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Table 1 Rhizobium species and their host genera*

Rhizobium species	Host genus	
	Generic name	Common name**
R. leguminosarum	Pisum, Vicia, Lathyrus, Lens	Pea, broad bean, sweet pea, lentil
R. trifolii	Trifolium	Clover
R. phaseoli	Phaseolus	Green bean
R. meliloti	Medicago, Melilotus, Trigonella	Alfalfa, melilot, fenugreek
R. lupini	Lupinus, Ornithopus	Lupin, serradella
R. japonicum	Glycine	Soybean

* adapted from Vincent (1977)
** common examples are given

The close taxonomic relatedness between pea (R. leguminosarum) and clover (R. trifolii) rhizobia is supported by many observations including ready genetic exchange between the two species (Johnston and Beringer, 1977), high DNA homology (Gibbins and Gregory, 1972), phage cross-specificity (Vincent, 1977) and similar carbohydrate utilization patterns (Graham, 1964). Thus results obtained with R. trifolii (used in this study) may often be applied to R. leguminosarum.

1.3 The process of nodule formation

1.3.1 Introduction

Mature nodules are highly specialized structures and the symbiosis is finely controlled with differentiation following an ordered sequence. Rhizobia infect root-hair cells and are enclosed in an infection thread. The thread then grows into the cortex of the root and induces division in a small group of inner-cortical cells directly ahead of the thread. After penetrating almost the entire cortex, the branches of the thread penetrate the meristematic area previously initiated in the inner-cortical cells. These cells are then invaded by the bacteria released from the infection thread, and subsequently differentiate into non-

dividing bacteroid-containing cells. As development proceeds, more cortical layers contribute to the nodule. The peripheral layers of the nodule, as well as its apical meristem, are not penetrated by the bacteria, but contain a vascular system.

Knowledge on nodule formation up to 1975 was reviewed by Dart (1977), and only recent progress is reviewed below. The terminology used for the range of symbiotic properties found in various legume-rhizobia interactions is described in Table 2.

Table 2 Terms used to describe the symbiotic property of a Rhizobium strain with respect to a specified host

Term (adjective)	Definition
Infective	Able to form nodules
Ineffective	Able to form nodules, but nodules do not fix nitrogen
Partially effective	Able to form nitrogen-fixing nodules, but the amount of nitrogen fixed is inadequate to support healthy plant growth
Effective	Able to form nodules that fix adequate nitrogen to support healthy plant growth

1.3.2 Recognition and adsorption

The recognition and attachment of compatible rhizobia is the first stage of the nodulation process (Dazzo et al., 1976). The basis for this host specificity has been the subject of much recent research concentrating on the role of plant lectins and rhizobial polysaccharides. The first indication that lectins may be involved in the specific recognition between the plant host and symbiont rhizobia was obtained by Bohlool and Schmidt (1974), who found that fluorescence-labelled soybean lectin bound to all but 3 of 25 R. japonicum strains tested, but did not bind to any of 23 other Rhizobium strains tested. Wolpert and Albersheim (1976) isolated lectins from the seeds of 4 species of legumes and found that the lectins bound to O-antigen containing lipo-polysaccharide (LPS) isolated from the homologous rhizobia, but not

that isolated from heterologous rhizobia. No binding of lectin to exo-polysaccharide in any combination was found. Albersheim et al. (1977) studied the LPS from different species of Rhizobium and from different strains of the same species and found that each strain possessed a structurally unique LPS, thus giving rise to enough diversity to account for the observed range of host specificity. Albersheim et al. (1977) also suggested that the lectins were enzymes capable of modifying the structure of the LPS of their symbiont, but not their non-symbiont rhizobia. Mutants of R. japonicum which were unable to nodulate soybeans were also found to lack an antigenic surface component associated with the O-antigen portion of the LPS, indicating a role for this portion in soybean nodulation (Maier and Brill, 1978). No experiments on binding to soybean lectin were reported. However, Bal et al. (1978), using the same strain of R. japonicum, found that ferritin-labelled soybean lectin bound only to the capsular polysaccharide, not the LPS, of approximately 1% of the bacterial population.

Bauer and coworkers (Bauer, 1977; Bhuvaneswari et al., 1977) found that the ability of R. japonicum strains to bind soybean lectin varied with the growth phase of the bacteria in defined medium. Most strains bound soybean lectin only when in the exponential phase of growth; however, other strains bound the lectin only when in the stationary, lag or early exponential phases. Thus binding to soybean lectin was found to be a transient rather than constitutive property of R. japonicum. Subsequent work (Bhuvaneswari and Bauer, 1978) demonstrated that the ability of R. japonicum strains to bind soybean lectin depended on the media in which they were cultured. While some strains bound the lectin irrespective of the media used for culture, other strains did not bind lectin unless they were cultured in the presence of soybean seedlings. Thus the soybean lectin receptors in the latter strains were synthesized specifically in response to the plant. These strains included those that Bohlool and Schmidt (1974) found did not bind lectin.

Meanwhile, Planque and Kijne (1977) reported that pea lectin bound to a non-O-antigenic polysaccharide found in R. leguminosarum cell walls, but which also occurred in the capsular but not exopolysaccharide. However, mutants of R. leguminosarum, which produced diminished amounts of exopolysaccharide but possessed normal LPS, were unable to nodulate peas (Sanders et al., 1978). No data on binding to pea lectin was included.

The recognition between R. trifolii and clover has been extensively studied. Clover root walls and the capsular polysaccharide of R. trifolii contained immunochemically unique but cross-reactive antigenic determinants which could bind to a 2-deoxyglucose-sensitive agglutinin present in seed extracts of clover (Dazzo and Hubbel, 1975). This agglutinin was proposed to be a multivalent lectin which functioned in recognition and adsorption by cross-bridging the similar surface polysaccharide receptors on R. trifolii and clover roots. The binding of R. trifolii to clover root hairs was sensitive to 2-deoxyglucose (Dazzo et al., 1976). Addition of 2-deoxyglucose eluted a protein, from the clover root, which was able to agglutinate R. trifolii but not other rhizobia (Dazzo and Brill, 1977). A lectin, called trifoliin, has been isolated from clover seeds, and was found to be concentrated at the root-hair region of clover seedlings where nodule formation occurred. It was eluted by 2-deoxyglucose and was able to specifically agglutinate R. trifolii (Dazzo et al., 1978). Trifoliin was suggested to fulfil the role postulated by Dazzo and Hubbel (1975). This research is the only work to show that the lectin examined is found on the roots and not just in the seed of the legume.

Nodulation by rhizobia is inhibited by fixed nitrogen (Fred et al., 1932). The binding of R. trifolii to root hairs and the concentration of trifoliin on the root-hair surface declined in parallel with increasing concentrations of fixed nitrogen, suggesting that the inhibition of nodulation by fixed nitrogen was mediated at this level (Dazzo and Brill, 1978).

In conclusion, the actual role of capsular, exo- and lipo-polysaccharides in determining the host range of Rhizobium remains to be clarified. It is not inconceivable that all three fractions are necessary for nodulation, and that lectin binding is only one of a series of complex steps involved in the recognition process. An interesting recent observation is the high-frequency transfer of nodulating ability from R. leguminosarum to other rhizobial species, suggesting that the host-specificity genes are plasmid-borne (Johnston et al., 1978a).

1.3.3 Subsequent steps

After recognition and adsorption, rhizobia penetrate into the root hair through the cell wall. The mechanism of penetration is unknown, but localized production of auxin at the root hair surface to soften the cell wall may be involved (reviewed by Dart, 1977). Penetration induces the formation of an infection thread which elongates, with continued division of rhizobia at the tip, until the target cell within the root cortex is entered. Rhizobia at the tip of the infection thread are then surrounded by membranes of plant origin (peribacteroid membranes) derived from the infection thread membrane (Robertson et al., 1978a); Tu, 1975) and budded off by an endocytotic process into the host cell cytoplasm. Robertson et al. (1978a) showed that Golgi bodies were involved in the synthesis of the infection thread walls and membranes (which are continuous with the plant cell walls and membranes) as well as peribacteroid membranes, and postulated that the Golgi body system was induced to synthesize these components as a consequence of infection. They cited evidence that wall-polymer synthesis in plants was under hormonal control at the level of the Golgi bodies, and suggested that, in view of the evidence that rhizobia produce auxins and cytokinins, the process of infection of plant cells involved hormonal control of the plant system of biosynthesis of cell walls and membranes by the invasive rhizobia. Hormone production may also be involved in other facets of nodule formation, including host cell endoreduplication (to form tetraploid cells) and division (Libbenga and Bogers, 1974; Syono et al., 1976; Dart, 1977).

After entrance into the cytoplasm, the bacteria and the peribacteroid membranes divide actively together. In some species, the bacteria also divide within the peribacteroid membrane (Libbenga and Bogers, 1974) which can be enlarged by fusion with cytoplasmic vesicles originating from the Golgi bodies (Robertson et al., 1978a). The host cells undergo enlargement once their division has ceased, which is usually at the time the bacteria enter their cytoplasm. In many species, the bacteria also enlarge to pleomorphic forms which may be up to 20 times the size of the original bacterium. The final result is infected tissue consisting of enlarged plant cells filled with altered bacteria, termed bacteroids, which have ceased dividing and are separated from the plant-cell cytoplasm by the peribacteroid membranes.

1.3.4 Leghaemoglobin synthesis and location

Mature nodules effective in nitrogen fixation are pink in colour, due to the presence of leghaemoglobin (legHb). LegHb is not formed by either legumes or rhizobia separately, but is confined to the bacteroid-containing tissue of the nodule wherein its synthesis probably precedes nitrogenase development (Godfrey et al., 1975). Both partners in the symbiosis participate in its synthesis. The globin portion of the molecule is genetically determined by the plant (Dilworth, 1969; Godfrey et al., 1975; Sidloi-Lumbroso et al., 1978), and is synthesized preferentially on the free polysomes in the host-cell cytoplasm (Godfrey et al., 1975; Verma and Bal, 1976). However, the haem portion of the molecule is synthesized by the bacteroids (Cutting and Schulman, 1969; Godfrey et al., 1975; Nadler and Avissar, 1977), and since free-living rhizobia do not synthesize large amounts of haem, an induction of the enzymes responsible for haem biosynthesis is required in the bacteroid.

The exact cellular location of legHb is controversial. Studies on its function (see Section 1.4.5) have assumed that it is localized in the space between the peribacteroid membrane and the bacteroid wall. This

location was supported by studies using 59 Fe-labelled nodules (Dilworth and Kidby, 1968), and was also suggested by cytochemical studies based on peroxidase staining (Bergersen and Goodchild, 1973; Truchet, 1972; Gourret and Fernandez-Arias, 1974). However, these studies were all based on the detection of Fe or haem, rather than of legHb itself. Verma and Bal (1976) attempted to localize legHb by using ferritin-conjugated antibodies to legHb, and looking at the location of the ferritin in sections of fixed tissue. Tissue slices were used in these experiments because the ferritin-antibody conjugates were unable to penetrate the cell-wall. They found that the ferritin was localized only in the plant cell cytoplasm and adjacent to the exterior of the peribacteroid membrane. Recently, Robertson et al. (1978b) isolated peribacteroid-membrane-enclosed bacteroids and found that they were devoid of legHb. No evidence for leakage of legHb during the isolation procedure was found, and thus the legHb was inferred to occur in the plant cell cytoplasm and not in the peribacteroid space.

1.4 Differences between bacteroids and cultured rhizobia

1.4.1 Viability

Until recently, bacteroid formation was thought to be a terminal differentiation in that reversion to vegetative forms never occurred (Bergersen, 1974). However, Sutton et al. (1977) found that lupin bacteroid viability was very sensitive to osmolarity, with over 50% of 11-day old bacteroids able to form colonies on media of suitable osmotic strength. This percentage declined with age, and by day 25 fewer than 1% were able to do so. The sensitivity of bacteroid viability to osmolarity was proposed to be due to a reversible change in the structure of the cell wall, which was followed by a second irreversible change in the older bacteroids (Sutton et al., 1977; Sutton and Mahoney, 1977). However, about 60-90% of clover bacteroids isolated from plant-cell protoplasts prepared from 6-week old nodules, and cultured in media of high osmolarity, were viable (Gresshoff et al., 1977). Bacteroids were isolated from protoplasts to avoid release of inhibitory substances from macerated plant tissues. However, Tsein

et al. (1977) reported that up to 90% of soybean bacteroids of various ages were viable although no specific precautions were taken during bacteroid isolation. In contrast to this, Gresshoff and Rolfe (1978) found that a high concentration (0.2 M) of mannitol in the medium was necessary if R. japonicum bacteroids were to form colonies. Nevertheless, all these data suggest that fully differentiated bacteroids retain the full capacity to lead an independent life.

Earlier reports suggested that a loss in bacteroid viability was associated with a decrease in DNA content (Dilworth and Williams, 1967; Bergersen, 1974). However, it is now accepted that bacteroids contain enough DNA for at least one complete genome (Sutton, 1974; Sutton and Robertson, 1974; Reijnders et al., 1975; Bisseling et al., 1977; Paau et al., 1977). A correlation between bacteroid size and DNA content was observed (Bisseling et al., 1977).

1.4.2 Ribosome and RNA content

Lupin bacteroids are greatly deficient in RNA and ribosomes (Dilworth and Williams, 1967; Sutton and Robertson, 1974) and retain less than 10% of the protein-synthesizing ability of cultured bacteria (Sutton and Robertson, 1974). The ability of bacteroids to incorporate amino acids into acid-stable material declines with their viability during nodule development (Sutton et al., 1977).

1.4.3 Cell walls and membranes

Early indications of the importance of the cell walls and membranes in the establishment of the symbiosis were obtained from antibiotic- or antimetabolite-resistant mutants which were ineffective as a result of changes in their cell walls or membranes (Schwinghamer, 1968; MacKenzie and Jordan, 1972; Pankhurst, 1974). Electron-microscopic examination showed that the bacteroid cell wall decreased in thickness, and developed a particulate surface during bacteroid development (MacKenzie et al., 1973). The membranes also underwent marked changes in protein composition (Sutton and Robertson, 1974).

Recent work has shown that bacteroids are more sensitive to lysozyme (van Brussel et al., 1977) and osmotic shock (van Brussel et al. 1977,

Gresshoff et al., 1977, Sutton et al., 1977) and contain less lipopolysaccharide in their cell wall than free-living rhizobia (van Brussel et al., 1977). In fact, the layer of murein between the inner and outer membrane almost completely disappears during bacteroid development (Robertson et al., 1978b). The inner membranes of the bacteroids are involved in electron supply to nitrogenase (Laane et al., 1978) and contain cytochromes not found in free-living rhizobia (Appleby, 1969; Robertson et al., 1978b). The peribacteroid membrane also undergoes changes in protein composition during bacteroid development (Robertson et al., 1978b).

1.4.4 Nitrogenase

That rhizobia only fix nitrogen in the bacteroid state was considered a dogma until 1974. Thereafter several groups reported nitrogenase activity in non-symbiotic rhizobia, at first in association with plant (not necessarily legume) cell cultures (Phillips, 1974; Child and LaRue, 1974; Child, 1975; Scowcroft and Gibson, 1975) and then completely independently from the plant, on agar (Kurz and LaRue, 1975; McComb et al., 1975; Pagan et al., 1975) and in liquid (Keister 1975; Tjepkema and Evans, 1975). Most strains able to fix nitrogen while free-living were slow-growing species, with one strain of R. leguminosarum (Kurz and LaRue, 1975) the only exception. Induction of nitrogenase in the absence of the plant in most fast-growing species while free-living is still awaited.

The conditions required for induction of nitrogenase include microaerophily, a fixed nitrogen source and an efficiently-utilized energy

source (Bergersen, 1977), and therefore resemble those found in bacteroids. (That bacteroids receive a supply of fixed nitrogen is suggested by the effective symbiotic properties of many amino acid auxotrophs (Denarie et al., 1976; Kondorosi et al., 1977a; this thesis)). The morphology of rhizobia fixing nitrogen on agar also resembles that of bacteroids (Pankhurst and Craig, 1978).

1.5 Biochemistry and physiology of nitrogen fixation

1.5.1 Structure of nitrogenase proteins

All nitrogenases so far studied consist of 2 component proteins. The smaller component, Component II (the Fe protein) has a mol. wt. of 51,000-69,000 daltons depending upon the organism from which it is isolated, and consists of 2 identical subunits. It contains 4 Fe and 4 acid labile S atoms per molecule (Eady and Smith, 1977). The larger component, Component I (the Mo-Fe protein) has a mol. wt. of ~220,000 with 4 subunits. The protein from Klebsiella pneumoniae contains 2 types of subunit of approximate mol. wt. 50,000 and 60,000 present in equal amounts (Eady et al., 1972; Kennedy et al., 1976). However, the Component I protein from R. japonicum (Israel et al., 1974) and R. lupini (Whiting and Dilworth, 1974) gave a single band when subjected to SDS disc electrophoresis of mol. wt. 50,000. Component I protein contains 1-2 Mo, 24-38 Fe and 16-28 acid labile S per molecule, depending on source and method of isolation (Eady and Smith, 1978). The bound Mo and Fe are released upon dissociation of the subunits.

There is some genetic and biochemical evidence that all molybdenum-containing enzymes contain a common molybdenum cofactor. For example, when acid-treated extracts of a variety of molybdoenzymes including Component I of nitrogenase were added to extracts of a nitrate reductaseless mutant of Neurospora crassa, nitrate reductase activity was restored (Nason et al., 1971). Further evidence for a Mo-containing cofactor was obtained by derepressing Azotobacter vinelandii for nitrogenase in medium containing tungstate instead of

molybdate. Inactive Component I was synthesized which could be activated in vivo by the addition of molybdate to the medium. Protein synthesis was not necessary for the activation (Nagatani and Brill, 1974). Activation of the inactive Component I was also observed in cell-free extracts if acid-treated Component I, from a variety of organisms, was added (Nagatani et al., 1974). Shah and Brill (1977) isolated a cofactor from Component I of A. vinelandii which was able to activate the inactive Component I synthesized in media containing tungstate instead of molybdate and could also activate Component I isolated from certain nitrogenase-less mutants. The cofactor contained Fe, Mo and S in the ratio 8:1:6 and was called FeMoCo. It contained approximately 50% of the Fe found in active Component I. Component I from A. vinelandii contained 2 Mo, 33 Fe and 27 acid-labile S per molecule, but it was not known whether one cofactor contained 2 Mo or if each Component I molecule contained 2 cofactors.

The FeMoCo from a variety of organisms are very similar, are extremely sensitive to oxygen and are unstable in aqueous environments (Shah and Brill, 1977). Since Component I is stable and active in aqueous environments, it may provide the FeMoCo with an aprotic environment. Nitrate reductase from N. crassa contains one haem Fe to one Mo and no non-haem Fe, and therefore does not contain the same cofactor (Shah and Brill, 1977).

Both component proteins of nitrogenase are sensitive to oxygen. Component I from R. japonicum (Israel et al., 1974) lost 50% of its activity on exposure to air for 4.5 min at 30° C, while Component II from R. lupini (Whiting and Dilworth, 1974) was extremely sensitive to oxygen, having a half-life of 1 minute.

1.5.2 General features of the nitrogenase reaction

A general outline of the features of the nitrogenase reaction is given in Figure 1.

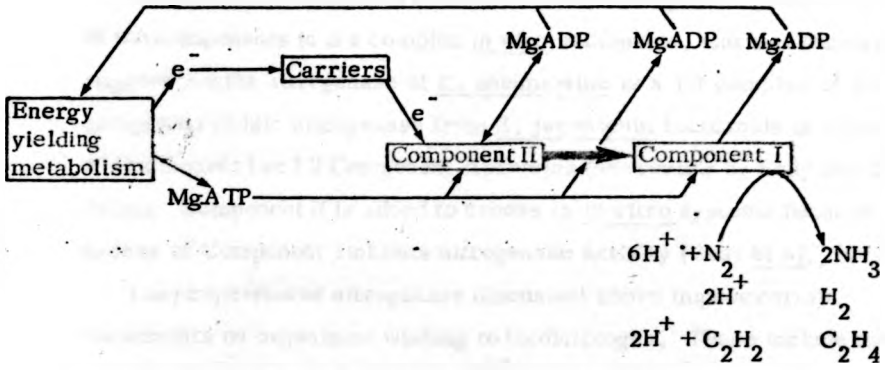

Fig. 1 Schematic representation of the general features of the nitrogenase reaction (adapted from Orme-Johnson et al., 1977).

Electrons are carried via low potential carriers (see later) to Component II which binds and subsequently hydrolyzes ATP. Component II functions as a very specific ATP-activated electron donor to Component I, and Component I binds and reduces the substrate. Component II is able to bind Mg.ADP and, when bound, MgADP blocks the reduction of Component II (Yates et al., 1975). Thus ADP is a potent inhibitor of nitrogenase activity. The ratio of ATP/ADP can thus control nitrogenase activity and in fact the reaction is appropriately sensitive over a physiologically reasonable range of ATP/ADP ratios (Haaker et al., 1974; Bergersen and Turner, 1973, 1975b; Davis and Orme-Johnson, 1976; Laane et al., 1978).

ATP plays a number of roles in the functioning nitrogenase complex. It induces a conformational change in Component II lowering the redox potential of the protein (Zumft et al., 1974). Furthermore, ATP hydrolysis is coupled to electron transfer from Component II to Component I (Eady et al., 1978b) and is also involved in complex formation between nitrogenase proteins and in the reduction of substrate by Component I (see reviews by Zumft and Mortenson (1975), Eady and Smith (1978) and Yates (1977)).

Functional nitrogenase is a complex of both components, but the ratio of the components in the complex in vivo is disputed. Current formulations suggest that the nitrogenase of K. pneumoniae is a 1:1 complex of the components while nitrogenase from R. japonicum bacteroids is a complex of 1 Component I and 2 Component II proteins (see review by Eady and Smith, 1978). Component II is added to excess in in vitro systems because an excess of Component I inhibits nitrogenase activity (Eady et al., 1972).

The properties of nitrogenase discussed above impose certain constraints on organisms wishing to fix dinitrogen. These include the supply of low potential electrons ($E^{o'} < -400$ mV), an active ATP-synthesizing system capable of maintaining a high ATP/ADP ratio, and the provision of an oxygen-free environment for the enzyme complex. These points will be discussed separately, with special emphasis on the Rhizobium-legume symbiosis.

1.5.3 Supply of electrons to nitrogenase

Ferredoxins and flavodoxins are the only known biological electron carriers able to transfer electrons to nitrogenase. Sodium dithionite is usually used as an artificial electron donor for in vitro experiments, and in fact the level of nitrogenase activity obtained with dithionite as reductant has not yet been achieved using physiological electron donors. This has been ascribed to loss of full in vivo potential during purification of the flavodoxins or ferredoxins (Yates, 1977).

The phosphoroclastic metabolism of pyruvate, which also yields ATP, is the most likely primary source of electrons for nitrogenase in Clostridium (see review by Dalton, 1974). Pyruvate is also able to donate electrons to nitrogenase when supplied to crude nitrogenase-containing extracts of K. pneumoniae. However, the sequence of electron transfer in the latter case is largely unknown (Yoch, 1974). In Clostridium, the sequence is pyruvate → ferredoxin → nitrogenase. Hydrogen can replace pyruvate in this sequence if hydrogenase is present (Walker and Mortenson, 1974) and, as well as possessing the classical bidirectional hydrogenase, C. pasteurianum possesses an unidirectional hydrogen-

oxidizing hydrogenase which can reduce ferredoxin (Chen and Blanchard, 1978).

NADH and NADPH are considered as the most likely primary electron donors to nitrogenase in aerobes. NADPH, but not NADH, was able to support nitrogenase activity in cell-free extracts of A. vinelandii, but only at very low levels unless NADPH-ferredoxin reductase from spinach was added (Benemann et al., 1971). There is other indirect evidence for NADPH being the primary electron donor to nitrogenase in aerobes. A mutant of A. vinelandii, which was unable to grow in nitrogen-free media when glucose was the sole carbon source, was able to grow if a tricarboxylic acid cycle intermediate was added (Mumford et al., 1959). Since NADP-linked isocitrate dehydrogenase (ICD) can constitute 1% of the soluble protein in A. vinelandii, the enzyme may be an important electron-donor to nitrogenase in this species (Chung and Franzen, 1969). Also, a correlation between ICD and nitrogenase activities was observed in pea bacteroids (Kurz and LaRue, 1977). The activity of the other tricarboxylic acid cycle enzymes did not show this correlation, suggesting to the authors that ICD provided the reductant for nitrogenase in the bacteroids.

On the basis of in vitro experiments, the scheme:

NADPH → ferredoxin → unknown intermediate → flavodoxin → nitrogenase was proposed for electron transfer to nitrogenase in A. vinelandii (Benemann and Valentine 1972) and in soybean bacteroids (Evans and Phillips, 1975). However, as the latter authors pointed out, the scheme suffers several limitations. Only very low levels of NADPH-ferredoxin reductase have been demonstrated in either species. Furthermore, when carrier-free illuminated chloroplasts were used as the source of reductant, ferredoxin was able to transfer electrons directly to nitrogenase, suggesting an alternative rather than sequential pathway of electron transport. A third criticism is that only a low level of nitrogenase activity was observed in the in vitro experiments from which the scheme was derived (Evans and Phillips, 1975; see also review by Yates, 1977).

The use of a pyridine nucleotide couple poses another problem. *In vitro* experiments have shown that half-maximal acetylene reduction rates are reached only when the potential has reached -450 mV. In terms of the standard potentials of NADP/NADPH, this means that the reduced to oxidized ratio of NADPH/NADP must reach a value in excess of 100:1 for nitrogen fixation to proceed efficiently (Benemann and Valentine, 1972; Orme-Johnson, 1977). However, Haaker *et al.* (1974) found that in *A. vinelandii* nitrogen fixation occurred at various NADPH/NADP ratios. They also found, by use of the uncoupler 4, -5, -6, 7-tetrachloro-2-trifluoro methylbenzimidazole and the fluorescence probe, 9-aminoacridine, that there was a direct correlation between the energized state of the membrane and nitrogenase activity. Since the uncoupler inhibited nitrogenase activity but had no effect on ATP levels, the authors argued that it affected electron transport. These results implied that an intact chemiosmotic gradient across the cytoplasmic membrane was necessary for nitrogenase activity. Such a requirement could explain the low nitrogenase activity of cell-free extracts of *A. vinelandii*. Lawne *et al.* (1978) recently extended these observations to *R. leguminosarum* bacteroids. They found, using an uncoupler, carbonylcyanide *m*-chlorophenylhydrazone, which specifically lowers the energized state of membranes, that the energized state and the integrity of the cytoplasmic membrane of the bacteroid were essential for nitrogen fixation to occur, and further that the supply of electrons *via* the energized state of the membrane played a part in the control of nitrogenase activity.

1.5.4 Requirement for ATP for nitrogenase activity *in vivo*

Many attempts have been made to estimate the number of moles of ATP required to fix one mole of nitrogen. *In vitro* experiments have suggested a value of 4-5 ATP molecules hydrolyzed per electron pair transferred, giving an apparent ATP/N_2 molar ratio of 12-15 (reviewed by Zumft and Mortenson, 1975). One approach used to estimate the *in vivo* requirement has been to compare the molar growth

yield of an organism grown on dinitrogen with that of the same organism grown on ammonia as sole nitrogen source. (A similar approach has been used with nodulated whole plants compared to plants grown with an inorganic fixed nitrogen source. Results obtained with the Rhizobium-legume symbiosis are discussed in Section 1.5.6). Estimates obtained using carbon-and energy-limited chemostat cultures range from 4-5 ATP/N_2 for A.chroococcum (Dalton and Postgate, 1969) to 29 ATP/N_2 for K. pneumoniae (Hill, 1976). However, Hill (1976) estimated that at least 45% of nitrogenase activity was utilized to evolve H_2, and that the true ATP/N_2 ratio was approximately 16. The value of Dalton and Postgate (1969) may be artificially low because of the large extrapolation made to eliminate ATP utilization attributed to 'respiratory protection'. They also assumed a Y_{ATP} value of 10.5 in the ammonia-assimilating population and that the amount of ATP generated from mannitol in the NH_4-assimilating and in the N_2-fixing populations was similar. The validity of these assumptions has been questioned (Dalton, 1974; Yates and Jones, 1974; Hill, 1976). For example, Hill (1976) found that a glucose-limited culture of K. pneumoniae grown on ammonia had a Y_{ATP} value of 10.5 whereas the Y_{ATP} value of a glucose-limited culture grown on N_2 was 4.5. However, Azotobacter species may consume a lower amount of carbohydrate than other species in order to produce ATP for nitrogenase because they possess an unidirectional hydrogenase capable of oxidizing, with the production of ATP, the H_2 evolved by nitrogenase (Hyndman et al., 1953; Dixon, 1972)(see also Section 1.5.7).

Anderson et al. (1977) studied the in vivo energy cost of N_2 fixation by using non-growing cells of a derepressed nif mutant strain of K. pneumoniae and correlating glucose consumption with ammonia and acetate production. The results indicated a minimal energy requirement of 21-25 ATP/N_2, including maintenance energy.

The rate of glucose consumption by *nif*⁻ strains was used to calculate maintenance energy and a corrected value of 14-16 ATP/N_2 was obtained. Using a strain devoid of hydrogen-producing systems other than nitrogenase, the authors also calculated that 1.3 mole H_2 were produced per mole of N_2 reduced, even under optimal conditions for nitrogen reduction. Taking this into account, an apparent ATP/2e⁻ value of 4 (equivalent to 12 ATP/N_2) was calculated. Nitrogenase also requires energy in the form of low potential electrons. The observed ratio of 0.65 H_2/NH_4 implied that at least 4.3 electron pairs were consumed per N_2 reduced. These electron pairs could theoretically give rise to 13 ATP, assuming a P/O ratio of 3, if used in oxidative phosphorylation, and therefore the total *in vivo* energy cost for N_2 fixation (including unavoidable H_2 production) in terms of ATP equivalents, was calculated to be close to 30 ATP/N_2 (Anderson *et al*, 1977).

Provision of ATP is only one part of the problem of ATP supply associated with nitrogenase. As stated earlier, the sensitivity of nitrogenase to inhibition by ADP may pose a real limitation on the functioning of nitrogenase under physiological conditions. The apparent K_m for ATP of nitrogenase is at least an order of magnitude higher than the apparent K_i for ADP. This suggests, assuming that the ATP+ADP pool in the vicinity of nitrogenase is constant, that nitrogenase will not function efficiently without 10 times more ATP than ADP. As also observed in free-living bacteria, the ATP/ADP ratio is important in the regulation of nitrogenase in bacteroids (Bergersen and Turner, 1975b); Appleby *et al*., 1976; Laane *et al*., 1978). Therefore, a high capacity ATP-generating system is required in the vicinity of nitrogenase in order to maintain a high ATP/ADP ratio and maximal nitrogenase activity.

1.5.5 Protection of nitrogenase against oxygen

Oxygen inhibits both activity and synthesis of nitrogenase (St. John *et al*., 1974; Eady *et al*., 1978a). Various mechanisms have been

evolved by different nitrogen-fixing organisms to overcome the problem of oxygen inhibition. These include avoidance, respiratory protection, conformational change, restriction to microaerophilic conditions, association with biological macromolecules, slime production, heterocyst formation and leghaemoglobin production (see reviews by Dalton (1974) and Yates (1977)). Only those mechanisms found in Rhizobium-associated nitrogen fixation will be discussed.

Leghaemoglobin (legHb) content and nodule effectiveness are closely associated in root nodules of legumes (Virtanen et al., 1947), although no similar protein was found in nodules formed by Rhizobium (cowpea sp.) on a non-legume (Coventry et al., 1976), formerly called Trema aspera (Trinick, 1973) but now identified as Parasponia parviflora (Akkermans et al., 1978).

The role of legHb was examined by studying the effect of oxygenated leghaemoglobin (legHb.O_2) on oxygen uptake and nitrogenase activity by bacteroid suspensions (Bergersen and Turner, 1975a). In vivo conditions were simulated by use of a system with no gas phase, in contrast to earlier experiments (Wittenberg et al., 1974). LegHb.O_2 allowed maximum rates of O_2 consumption and nitrogenase activity at levels of O_2 as low as $\frac{1}{25}$ of that which was limiting in the absence of the carrier. The authors suggested that the role of legHb was to maintain a high flux but low concentration of O_2 at the bacteroid surface, in a range which was optimal for ATP production but did not cause inhibition of nitrogenase activity. This range of O_2 concentration was found to be optimal for ATP production by a terminal oxidase system which had a high affinity for O_2 and which produced ATP efficiently (Bergersen and Turner, 1975b). Cytochrome P_{450} was found to act as an intracellular O_2 carrier from legHb to this high oxygen-affinity terminal oxidase system (Appleby et al., 1975). However, the inability to find a protein similar to legHb in the equally active nodules formed by Rhizobium (cowpea sp.) on Parasponia parviflora (Coventry et al., 1976) suggests that these nodules, at

least, possess another mechanism for the protection of nitrogenase from oxygen damage. The authors postulated that an oxy-polyphenol oxidase may act as an alternative O_2 carrier to maintain a stable low level of O_2.

Leghaemoglobin was therefore thought to transport O_2 from the peribacteroid membrane to the bacteroid. However, recent evidence (Section 1.3.4) has suggested that legHboccurs in the plant cytoplasm only. Its role may therefore be to transport O_2 from the plasma membrane through to the peribacteroid membrane (Robertson et al., 1978b). If this is the case, a considerable barrier to oxygen diffusion still exists in the peribacteroid space.

Leghaemoglobin has been found only in legume nodules and both partners in the symbiosis participate in its synthesis (see Section 1.3.4). Therefore, free-living nitrogen-fixing rhizobia must protect their nitrogenase from inactivation by a different mechanism. This problem has been studied by Bergersen and co-workers (Bergersen et al., 1976; Bergersen, 1977) who found that nitrogenase activity was optimal under microaerophilic conditions. Use of succinate in the medium allowed nitrogen fixation at higher oxygen concentrations presumably due to respiratory protection of nitrogenase. Rhizobia produce large quantities of gum, and this may also function in the restriction of oxygen supply to the bacteria. Evidence that the oxygen level was also critical to nitrogenase activity in Rhizobium grown on plates in air was presented by Pankhurst and Craig (1978). They found that nitrogenase activity developed only in cells at a certain depth into a soft-agar layer, and that this depth changed with the oxygen concentration in the atmosphere above the agar during growth.

1.5.6 Energy requirements for legume symbiotic nitrogen fixation

Fred et al. (1932) stated that the co-ordination between carbohydrate synthesis and nitrogen fixation has been pointed out by several workers, and quoted Schindler (1885) as one of the earliest. Several studies since that time have served to emphasize the importance of photosynthesis as a factor controlling the rate of nitrogen fixation and

to estimate the amount of energy required for nitrogen fixation in the legume nodule.

The importance of photosynthesis was inferred from various physiological experiments wherein a change in the supply of photosynthate to the nodule was correlated with a change in nitrogen fixation (Allison, 1935; Butler et al., 1959; Lawn and Brun, 1974; Streeter, 1974; Gibson, 1976; Ham et al., 1976; Sprent, 1976; Bethlenfalvay and Phillips, 1977a).

CO_2 enrichment experiments have provided the most dramatic demonstration of the limitation of nitrogen fixation by photosynthate supply. The effect of increased CO_2 concentration in the foliar canopy is to decrease photorespiration and thus provide an increased supply of photosynthate. Enrichment of soybean plants from 25 days old to maturity with a three-fold concentration of CO_2 led to an increase in the amount of nitrogen fixed from an unenriched value of 75 Kg hectare^{-1} to 425 Kg hectare^{-1}. This indicated that the enriched plants fixed 85% of their nitrogen requirement compared to only 25% by unenriched plants. In addition, the total nitrogen input was increased from 295 to 510 Kg hectare^{-1}. The increased nitrogen fixation resulting from CO_2 enrichment was due to three effects: (i) an immediate doubling of nitrogen-fixing activity per mass of nodules, suggesting that there was an excess of nitrogenase in the nodules; (ii) a doubling in the number of nodules; (iii) an extended period of nitrogen fixation (Hardy and Havelka, 1976). Short-term CO_2 enrichment studies also revealed large increases in acetylene reduction by pea nodules at a time corresponding to the arrival of the photosynthate produced under the CO_2 enrichment conditions (Phillips et al., 1976). A study of the effect of light intensity on nitrogen fixation provided further evidence that nitrogen fixation rate was dependent on the concurrent CO_2 reduction rate (Bethlenfalvay and Phillips, 1977a).

The data presented above imply that it is the supply of photosynthate that limits nitrogen fixation in the nodule, rather than the amount of nitrogenase present, etc. The data also suggest that

nitrogen fixation by legumes is dependent upon an immediate supply of photosynthate, rather than upon storage carbohydrate, although soybean bacteroids do store large amounts of poly-8-hydroxy butyrate (PHB) (Wong and Evans, 1971). The diurnal fluctuations found in legume nitrogen fixation provide further support for this hypothesis (Minchin and Pate, 1974). The results of Hardy and Havelka (1976) also suggest that the plant can respond to increased photosynthate availability by increasing the number of nodules formed, thereby increasing the amount of nitrogenase.

Several approaches have been used to estimate the amount of carbohydrate consumed in symbiotic nitrogen fixation by legumes. Earlier studies were based on comparing the dry matter and nitrogen accumulation of nodulated plants utilizing atmospheric nitrogen with that of non-nodulated plants provided with an equivalent amount of fixed nitrogen (Bond, 1941; Gibson, 1966; Minchin and Pate, 1973). However, this method suffers several limitations. Substantial physiological and morphological differences were found between plants utilizing nitrate and those fixing nitrogen. For example, nitrate-fed plants had greater leaf areas, shoot chlorophyll contents, and increments of root dry-weight and nitrogen, but lower shoot dry-weight increments than did nodulated plants (Minchin and Pate, 1973). Nevertheless, all three studies indicated an approximate equivalence of carbohydrate consumed, whether nitrate or dinitrogen was the nitrogen source utilized (Bond, 1941; Gibson, 1966; Minchin and Pate, 1973). However, recently Silsbury (1977) suggested that symbiotic nitrogen fixation required more energy than nitrate assimilation.

Minchin and Pate (1973) also followed the fate of net photosynthate produced over a 9 day period of exponential growth of nodulated pea plants. They found that 32% of the photosynthate was translocated to the nodules, of which 16% was consumed for growth, 37% in respiration and 47% returned to the shoot as products from nitrogen fixation. This indicated that the nodules required for growth, respiration and export 4.1 mg C (≡ 10.3 mg carbohydrate) per mg N fixed.

A study of cowpeas showed that their nodules consumed 9% of the C from the plant's net photosynthate, of which 43% was respired, 6% made into dry matter and 51% returned to the shoot as fixation products. This data indicated that 6.8 mg C were consumed by the nodule for every mg N fixed (Herridge and Pate, 1977).

Mahon (1977a, b) adopted a different approach. By considering total respiration as the sum of several additive components, he derived the following equation:

$$R = RMW + RG\,(dW/dt + RF\,(N_2\text{-ase})),$$

where R was the total respiration of nodules plus roots, W was nodule plus root dry weight, N_2ase was nitrogenase activity, and RM, RG and RF were the maintenance, growth and fixation coefficients, respectively. He also found that the addition of NH_4NO_3 to nodulated plants decreased the respiration associated with nitrogenase activity while causing little difference in growth and maintenance components when compared to N-free treated plants of similar age (Mahon, 1977a). This surprising conclusion was derived from the fact that the regression coefficient between CO_2 evolved and acetylene reduced was relatively constant, regardless of the treatment applied, i.e. the regression coefficients found between acetylene reduction and CO_2 evolution in the presence and absence of 15 mM NH_4NO_3 at three different light intensities were similar (0.23 ± 0.04) (Mahon, 1977b). From this data, a value of 17 mg carbohydrate utilized per mg N fixed was calculated.

It is worth noting that the above studies (Minchin and Pate, 1973; Mahon, 1977a, b) measured respiration as CO_2 evolution. Recent work has shown that nodules possess an active phosphoenolpyruvate carboxylase (Lawrie and Wheeler, 1975; Christeller et al., 1977) and thus some of the CO_2 released through respiration might be captured by the carboxylase, and hence CO_2 evolution may provide an underestimation of the amount of respiration. Nevertheless, it is clear that nodule nitrogen fixation requires a sizeable portion of the plant's net photosynthate. The exact energy requirements of nodule

nitrogenase in terms of ATP and reductant cannot, however, be calculated, because the actual carbon source received by the bacteroids, wherein nitrogenase functions, is unknown. Pathways of carbohydrate catabolism available to the bacteroid, and to free-living rhizobia, are also only poorly understood. Current knowledge of this subject is discussed in Section 1.6.

1.5.7 Hydrogen metabolism in Rhizobium and nodulated legumes

Since energy supply is a major factor limiting symbiotic nitrogen fixation, any factor which increases the energy available to the bacteroid should increase the productivity of symbiotic nitrogen fixation. The ATP-dependent production of hydrogen by nitrogenase represents a waste of energy, and Schubert and Evans (1976) reported that nodules formed by many Rhizobium-legume associations used 30-60% of their total electron flux through nitrogenase for hydrogen production. However, a few symbionts evolved little or no hydrogen in air.

Dixon (1967) found that some pea nodules did not evolve H_2 but in fact were able to take up H_2, and oxidize the hydrogen produced by nitrogenase. These nodules possessed an unidirectional H_2-oxidizing hydrogenase (Dixon, 1972) similar to that found in Azotobacter (Hyndman et al., 1953; Dixon, 1972). The oxidation of hydrogen in the pea nodules was coupled to ATP synthesis, presumably via oxidative phosphorylation (Dixon, 1972). The capacity of a nodule to recycle rather than evolve the hydrogen produced by nitrogenase is a property of the rhizobial strain used for inoculation (Dixon, 1972; Schubert et al., 1977; Carter et al., 1978) and the Rhizobium strains which form nodules that do not evolve hydrogen can express hydrogenase activity while free-living if cultured under specific conditions (Maier et al., 1978a, b).

All studies so far (Schubert et al., 1977; Carter et al., 1978; Maier et al., 1978a, b) have failed to find a significant correlation between the presence of hydrogenase and improved symbiotic performance, i.e.

improved acetylene reduction rate or plant dry-weight. Such a correlation would have been expected if symbiotic nitrogen fixation is energy-limited, as is presently thought. Also only Dixon (1972) has shown that hydrogen oxidation is coupled to ATP synthesis in bacteroids and the physiological electron acceptor of hydrogenase has not been identified. Nevertheless, large-scale field-tests designed to test the agronomic importance of the hydrogenase have not been reported and so hydrogenase possession may yet prove to be significant.

1.6 Carbohydrate metabolism in Rhizobium

1.6.1 Introduction

The production of ATP and reductant from carbohydrate catabolism is obviously a fundamental process in the mature nodule. Considering the importance of this process, very little is known about the basic physiology of carbohydrate metabolism, either in the nodule or in free-living rhizobia. One of the main aims of this study was to acquire basic knowledge on carbohydrate catabolism in R. trifolii and by the isolation of mutants of R. trifolii defective in carbohydrate catabolism, to delineate the contribution of the bacteria to carbohydrate catabolism in the nodule. The next two sub-sections review knowledge gained up to 1976 on carbohydrate catabolism by bacteroids and by free-living rhizobia. Knowledge gained since 1975 is discussed in Sections 6.4 and 6.5.

1.6.2 Carbohydrate metabolism by bacteroids

Sucrose is the major product of photosynthesis translocated to the nodules (Bach et al., 1958) wherein its concentration has been correlated with acetylene reduction (nitrogen fixation) rate (Streeter and Bosler, 1976). However, the nodule cytosol contains an active invertase (Kidby, 1966, 1967; Robertson and Taylor, 1973) and the actual carbon source received by the bacteroid and utilized to provide the large amount of ATP and reductant required for nitrogen fixation is unknown.

Succinate was found to be the best substrate for the support of O_2-linked respiration by aerobically-isolated bacteroids (Tuzimura and Meguro, 1960; Jordan, 1962) and to be the best substrate for enhancement of nitrogen fixation in anaerobically-prepared bacteroids; fumarate and pyruvate were found to be less effective while sucrose was ineffective (Bergersen and Turner, 1967). Neither sucrose nor glucose was able to support O_2-linked respiration in bacteroids (Tuzimura and Meguro, 1960; Kidby, 1967), a result attributed to damage of a glucose transport mechanism during bacteroid preparation (Kidby, 1967). Rigaud et al. (1973) did find stimulation of acetylene reduction by glucose with similar maximum levels reached as with succinate. However, under anaerobic conditions, the optimal concentration of glucose was 120 mM whereas succinate was most effective at 25 mM with higher concentrations causing a sharp fall-off in acetylene reduction. The authors suggested that, because of the high optimum concentrations, glucose exerted its effect by providing an osmoticum, allowing conservation of endogenous substrates. Such an effect had been previously reported for sucrose (Bergersen and Turner, 1967). Nevertheless, criticisms of these results on the grounds of damage to bacteroid membranes seem particularly valid in view of the recent evidence that such damage readily occurs (Gresshoff et al., 1977; Sutton et al., 1977; Laane et al., 1978). Also, the above work was done with slow-growing species which are unable to grow on sucrose (Graham, 1964) and probably do not possess invertase (Martinez de Drets et al., 1974). Fast-growing species can grow on sucrose and do possess invertase (Martinez de Drets et al., 1974).

Enzymes characteristic of the pentose-phosphate (PP) pathway (glucose-6-phosphate (G6P) dehydrogenase and 6-phosphogluconate (6PG) dehydrogenase) and the Embden-Meyerhof-Parnas (EMP) pathway (fructose diphosphate aldolase and phosphofructokinase) have been found in bacteroids (Kidby, 1967). Wong et al. (1971)

also reported G6P dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase in bacteroids. These experiments were done with bacteroids formed by slow-growing strains of rhizobia; similar experiments using fast-growing strains are lacking. However, hexoses are still proposed as the main substrates used by bacteroids *in vivo* (Bergersen, 1974, 1977).

1.6.3 Central carbohydrate metabolism in free-living rhizobia

Pathways of central carbohydrate metabolism have not been studied in bacteroids, apart from the enzyme activities mentioned above, and relatively little is known about the pathways in free-living rhizobia. Both fast-growers (Katznelson and Zagallo, 1957) and slow-growers (Keele *et al.*, 1969) possess the Entner-Doudoroff (ED) pathway. Enzymes of the EMP pathway (fructose diphosphate aldolase and phosphofructokinase) were reported in fast-growing species (Katznelson and Zagallo, 1957); however, Martinez de Drets and Arias (1972) were unable to find significant levels of fructose diphosphate aldolase in any species of *Rhizobium*. Keele *et al.* (1969), on the basis of radiorespirometric and enzymatic data, concluded that *R. japonicum* catabolized glucose primarily by the ED pathway. The key enzyme of the PP pathway, 6PG dehydrogenase, was absent (Keele *et al.*, 1969). Gluconate catabolism was similar to glucose catabolism. However, a minor pathway, leading to 2-oxoglutarate *via* 2-keto- and 2,5-diketogluconate, was suggested to fulfil an anaplerotic role (Keele *et al.*, 1970). Fast-growing species do possess the NADP-dependent 6PG dehydrogenase (Katznelson and Zagallo, 1957; Martinez de Drets and Arias, 1972) which is absent from slow-growing rhizobia (Keele *et al.*, 1969, 1970; Martinez de Drets and Arias, 1972) and hence have the additional potential to use the PP pathway for hexose catabolism.

Metabolism of the pyruvate produced by the ED, EMP or PP pathways requires a functional TCA cycle in *Rhizobium* (Keele *et al.*, 1969, 1970) and hence an anaplerotic pathway is required for growth to be maintained (Kornberg, 1966). The glyoxalate cycle operates only when the rhizobia are cultured on a fatty acid and is not present in bacteroids (Johnson *et al.*, 1966). Phosphenolpyruvate carboxylase

has been reported in nodules (Lawrie and Wheeler, 1975) but it is confined to the nodule cytosol (Christeller et al., 1977). Lillich and Elkan (1971) suggested that oxaloacetate can be formed directly from pyruvate by R. japonicum but presented no enzymatic data. Hence, the nature of the anaplerotic enzyme(s) used by Rhizobium for growth on sugars is unknown.

Reports on enzyme activities do not prove that a pathway is physiologically significant. Therefore the only solid conclusion that can be drawn from the above data is that R. japonicum metabolizes glucose by the ED pathway (Keele et al., 1969). This, however, has been recently challenged by Mulongoy and Elkan (1977a) who suggested that the EMP and ED pathways operate simultaneously in the strain of R. japonicum which was used by Keele et al. (see also Sections 6.3 and 6.4). The involvement of the tricarboxylic acid cycle is, however, unquestioned.

1.7 Nitrogen assimilation and its regulation

1.7.1 Introduction

Both plants (Miflin and Lea, 1976) and bacteria assimilate inorganic nitrogen into organic compounds at the level of ammonia by one of two pathways. The first is catalyzed by glutamate dehydrogenase (GDH) (reaction 1) and the second by glutamine synthetase (GS) and glutamate synthase (GOGAT) (reactions 2 and 3).

Reaction 1: 2-oxoglutarate + NH_3 + NADPH → glutamate + NADP

Reaction 2: glutamate + NH_3 + ATP → glutamine + ADP + Pi

Reaction 3: 2-oxoglutarate + glutamine + NAD(P)H → 2(glutamate) + NAD(P)

Pathway 2: (sum of reactions 2 and 3):

2-oxoglutarate + NH_3 + NAD(P)H + ATP → glutamate + NAD(P) + ADP + Pi

Both pathways yield the same net result, but the second is less energetically favourable in that 1 mole of ATP is hydrolyzed per mole of glutamate formed.

The pathway used depends upon both the organism and the nitrogen source (Tempest et al., 1973). In general, however, the GS/GOGAT

pathway is used when the concentration of ammonia in the medium is low, while GDH is used only at high ammonia concentrations. This is reflected by the fact that GDH has a high K_m for ammonia whereas GS has a low K_m for ammonia and GOGAT a low K_m for both 2-oxoglutarate and glutamine.

Mutants of K. pneumoniae which lack GOGAT are unable to grow if the concentration of ammonia in the medium is less than 1 mM, or if one of various amino-acids or dinitrogen is the sole nitrogen source present (Nagatani et al., 1971). In enteric bacteria, at least, ammonia represses the synthesis of enzymes required for utilizing these compounds (histidase: Prival and Magasanik, 1971; nitrogenase: Tubb and Postgate, 1973) and this repression is mediated by the intracellular level of GS (histidase: Prival et al., 1973; nitrogenase: Streicher et al., 1974; Tubb, 1974). The level of GS is high when the concentration of ammonia is low, or histidine or dinitrogen is the sole nitrogen source. Conversely, the level of GS is low when the concentration of ammonia is high, irrespective of other nitrogen sources present. The histidase (Prival et al., 1973) and nitrogenase (Streicher et al., 1974; Tubb, 1974) in mutants which produce GS constitutively are insensitive to repression by ammonia. However, the oxygen-regulation of nitrogenase synthesis is independent of GS (Eady et al., 1978a).

Both the activity and the synthesis of GS in enteric bacteria are repressible in vivo. When bacteria grown in a medium containing a low concentration of ammonia are removed to a medium containing a high concentration of ammonia, their GS rapidly becomes adenylylated and this form of the enzyme is biosynthetically inactive. If the bacteria are returned to a medium containing a low concentration of ammonia, the GS is rapidly deadenylylated. The adenylylation/deadenylylation reactions are controlled by a complex cascade system (Ginsburg and Stadtman, 1973; Wohlheuter et al., 1973) in response to the intracellular ratio of 2-oxoglutarate to glutamine, which is a very sensitive signal for the availability or lack of ammonia (Ginsburg

and Stadtman, 1973; Foor et al., 1975; Senior, 1975). Mutants of K. aerogenes which are unable to adenylylate GS constitutively synthesize active GS, whereas mutants unable to deadenylylate it produce very low amounts, suggesting that adenylylated GS inhibits the transcription of the GS structural gene (Foor et al., 1975). Unadenylylated GS is able to activate the in vitro transcription of the genes required for histidine utilization (hut operon) (Tyler et al., 1974). Therefore GS plays a positive role in the regulation of nitrogen-assimilatory enzymes as well as its role in ammonia assimilation per se.

1.7.2 Ammonia assimilation in symbiotic and free-living rhizobia

Since one aim of this study was to investigate nitrogen assimilation in R. trifolii, only information published prior to 1976 is reviewed here. The extensive literature published since 1975 is reviewed in Sections 5.4 and 5.5.

Ammonia is the initial product of symbiotic nitrogen fixation as befits a process catalyzed by nitrogenase (Bergersen, 1965). Contradictory reports have appeared concerning the presence of the GS/GOGAT pathway in bacteroids. Assays of GS using the transferase assay, which measures both adenylylated and unadenylylated enzyme, showed appreciable activity in bacteroids (Dunn and Klucas, 1973; Kurz et al., 1975). However, assays using the biosynthetic assay, which measures unadenylylated enzyme only (however, note that adenylylation/deadenylylation of rhizobial GS has not yet been proved) found only very low GS activities in bacteroids which were insufficient to account for assimilation of the ammonia produced by nitrogenase (Brown and Dilworth, 1975; Robertson et al., 1975a). High levels of biosynthetic GS activity have been found in the plant fraction of nodules (Dunn and Klucas, 1973; Brown and Dilworth, 1975; Robertson et al., 1975a) and in fact the amount of GS activity in the nodule cytosol, but not in the bacteroid, increased markedly in parallel with the induction of

nitrogenase (Robertson et al., 1975a). However, no workers except Kurz et al. (1975) have measured adenylylated and unadenylylated GS in the same sample of bacteroids. The latter authors measured the adenylylation state of GS only at a time when the nitrogenase activity of the nodules had declined (after flowering) and found that it was highly adenylylated.

Glutamate synthase has been found in bacteroids from the nodules of many legumes (Nagatani et al., 1971; Dunn and Klucas, 1973; Ryan and Fottrell, 1974; Brown and Dilworth, 1975; Robertson et al., 1975b) and in some it was present in amounts sufficient to account for incorporation of the ammonia produced by nitrogenase (Dunn and Klucas, 1973; Ryan and Fottrell, 1974; Robertson et al., 1975b). However, a 10-fold increase in GOGAT activity occurred in the nodule cytosol during nodule development, whereas the amount of the enzyme in the bacteroids remained constant (Robertson et al., 1975b). Other workers were unable to find GOGAT activity in the nodule cytosol fraction (Dunn and Klucas, 1973; Ryan and Fottrell, 1974; Brown and Dilworth, 1975), but this may be due to the assay procedures used because Robertson et al. (1975b) found that the enzyme was both unstable and inhibited by amino-acids present in the nodule cytosol.

In general, only low levels of biosynthetic GDH were found in either bacteroids or the nodule cytosol. It is thought that the majority of the ammonia produced by nitrogenase in the bacteroid is assimilated by plant enzymes in the cytosol (Brown and Dilworth, 1975; Kurz et al., 1975; Robertson et al., 1975a, b).

Brown and Dilworth (1975) also examined ammonia assimilation in free-living R. japonicum, R. leguminosarum and R. trifolii under chemostat culture. When ammonia was the limiting substrate, GS and GOGAT were relatively high in all three species. Biosynthetic GDH was low in R. japonicum and R. trifolii, but in R. leguminosarum it was present in similar amount to that found under glucose limitation.

When under glucose limitation, all three species possessed biosynthetic GDH but GS was not detected in any. Rhizobium trifolii and R. japonicum possessed GOGAT under these conditions, but the activity had disappeared in R. leguminosarum. These fluctuations were therefore similar to those found in enteric bacteria.

The findings discussed above pose intriguing questions. Why do bacteroids overproduce ammonia which is excreted into the nodule cytosol ? Are rhizobial GS or GOGAT involved in the regulation of the synthesis or activity of nitrogenase ? Do rhizobial GS or GOGAT participate at all in the assimilation of ammonia produced by nitrogenase, and how are they regulated? Several aspects of these problems could be clarified by the use of appropriate rhizobial mutants.

1.8 The use of mutants to study the Rhizobium-legume symbiosis

The establishment of an effective symbiosis between Rhizobium and its host legume involves the regulated differentiation of both partners (Section 1.3). Thus, to dissect the process, a combined physiological, biochemical and genetic approach is required. The study of independent mutants of each partner has revealed some information on their respective roles, and of factors important in the symbiosis. The plant-genetic studies have been reviewed by Caldwell and Vest (1977) and only information gleaned from the study of Rhizobium mutants is included in this section. Knowledge prior to 1974 on the symbiotic consequences of mutation to antibiotic or antimetabolite resistance and to auxotrophy has been reviewed (Denarie et al., 1976; Schwinghamer, 1977). The use of mutants to study the role of Rhizobium in recognition and adsorption and to reveal the importance of the cell-wall and membrane in the symbiosis was discussed earlier (Sections 1.3.2 and 1.4.3).

The symbiotic properties of various mutants resistant to antibiotics, etc. have been described, but the target sites of the actual

mutations were not identified. Thus, for example, up to 50% of mutants resistant to some antibiotics (novobiocin, penicillin, vancomycin) were ineffective (Schwinghamer, 1964, 1967) but the difference between these and the other effective 50% was not reported. In general, however, mutants resistant to antibiotics which inhibit cell wall-cell membrane biosynthesis are often ineffective, whereas mutants resistant to inhibitors of protein synthesis show no alteration in symbiotic properties (Denarie et al., 1976; Pankhurst, 1977; Schwinghamer, 1977). Exceptions to the last class are mutants resistant to viomycin, kanamycin and neomycin, where resistance may be due to altered cell wall permeability, restricting the entry of the antibiotic, at least in the case of viomycin (MacKenzie and Jordan, 1972). Resistance to viomycin invariably causes ineffectiveness in fast-growing strains (Schwinghamer, 1967; MacKenzie and Jordan, 1972; Pankhurst, 1977) but has no effect on the symbiotic properties of slow-growing strains (Levin and Montgomery, 1974; Pankhurst, 1977). This may be a reflection of either a different mechanism of resistance in fast- and slow-growers, or a different role of the cell wall in the symbiosis formed by fast- and slow-growers. For example, as stated earlier, the murein layer of the cell envelope almost completely disappears during bacteroid development in the slow-growing R. lupini (Robertson et al., 1978b).

Auxotrophic mutants are generally effective, exceptions being purine, pyrimidine and leucine negative mutants (Denarie et al., 1976)(Table 3). Results overall suggest that the plant is able to supply the rhizobia with adequate levels of amino-acids at all stages of the symbiosis. Studies on a riboflavin-negative mutant of R. trifolii showed that addition of riboflavin allowed effective nodulation to occur (Schwinghamer, 1970). Bacteroid development was arrested unless the extra riboflavin was supplied (Pankhurst et al., 1972) and it was found that bacteroids contained 18 times more flavin adenine dinucleotide than cultured rhizobia (Pankhurst et al., 1974).

Table 3 Association between nutritional requirements and symbiotic properties of strains of Rhizobium

Rhizobium: species and strains	Host plant: species and cultivars	Nutritional requirements of mutants	No. of mutants	Symbiotic properties* E	PE	I	NN
R. leguminosarum L4	Pisum sativum Home freezer Freezonian	Adenine + thiamin	1				1
						1	
R. trifolii	Trifolium pratense (Kenland)	Riboflavin	1				
						1	
	Trifolium subterraneum (Mt Barker)			1			
R. meliloti	Medicago sativa						
Sa 10	Du Puits	Adenine	1			1	
2011		Adenine	2			2	
		Uracil	1			1	
		Glycine	3	3			
		Methionine	2	2			
		Cysteine	2	2			
L5-30	Du Puits	Adenine	10	1	1	8	
		Uracil	2			2	
		Thiamine	2	2			
		Methionine	8	7	1		
		Cysteine	2	2			
		Glycine	1	1			
		Arginine	3	3			
		Ornithine	8	7	1		
		Tryptophan	1	1			
		Phenylalanine	4	4			
		Leucine	11		2(lk)	9	
		Isoleucine + valine	20	4	6	10	
		Isoleucine + valine + leucine	3			3	

*E, effective; I, ineffective; PE, partly effective; NN, non-nodulating; lk, leaky auxotroph.

** adapted from Denarie et al. (1976).

The significance of the invariable association between adenine requirement and ineffectiveness is discussed in Section 3.3.

The influence of the host plant on bacteroid development has been demonstrated by the fact that the symbiotic properties of some mutants are dependent upon the host cultivar on which they are tested (Pankhurst et al., 1974; Pankhurst, 1977). For example, the riboflavin-negative mutant of R. trifolii was ineffective on some cultivars of subterreanean clover but fully effective on others. This was not related to the flavin content of the roots of these cultivars (Pankhurst et al., 1974).

Mutants described thus far have involved the selection of 'in vitro' markers and their correlation with 'in vivo' symbiotic properties. The lesions are probably not in pathways specifically involved in the development and functioning of the symbiosis, and the symbiotic defect may in fact be a trivial consequence of the selected phenotype. Before the work described in this thesis was begun, only one study of mutants with a lesion that may be directly involved in the symbiosis had been reported. One class of nitrate-reductase-deficient mutants of R. meliloti formed an ineffective symbiosis. This was suggested to be evidence for a common genetic determinant of nitrogenase and nitrate reductase such as a molybdenum cofactor (but see Section 1.5.1), but the specific lesion in the mutants was not identified (Kondorosi et al., 1973). Subsequent work (Sik and Barabas, 1977) found that the mutants did possess assimilatory nitrate reductase but lacked a 'regulatory' nitrate reductase formed in aerated media containing casamino acids. The mutants were able to form a normal symbiosis if purine, riboflavin, glutamate, aspartate, arginine or ornithine was added. It was suggested that these compounds assisted in the removal of ammonia, which otherwise inhibited nitrogenase, from the vicinity of nitrogenase. In bacteroids formed by wild-type rhizobia, the ammonia would be removed by oxidation to nitrate, which would then induce the 'regulatory' nitrate reductase.

Thus, a regulatory connection between the nitrate reductase and nitrogenase was suggested (Sik and Barabas, 1977). This explanation seems tenuous. Pagan et al. (1977) found that only 2 from 48 nitrate reductase mutants of cowpea rhizobia were defective in nitrogenase in vitro. Of these two, one was unable to nodulate and the other was comparatively slow in establishing an effective symbiosis, though levels of acetylene reduction comparable to wild-type were achieved by 3 weeks after inoculation. The other nitrate reductase mutants were effective, and no evidence for a role for nitrate reductase in the symbiosis or for a common subunit between nitrate reductase and nitrogenase was found (Pagan et al., 1977).

Isolation of mutants defective only in symbiosis (and therefore only detectable by plant tests) has been reported since this study was begun. Maier and Brill (1976) found five such mutants, of which two were non-nodulating (and have since been found to have altered O-antigen; Maier and Brill, 1978), and three were ineffective. Leghaemoglobin was not produced in nodules formed by two of these mutants, but the third did produce pink nodules and was found to lack component II of nitrogenase. Biochemical lesions were not identified in the other two nodulating mutants, but one was also unable to fix nitrogen when free-living. Beringer et al. (1977) isolated ineffective mutants of R. leguminosarum by direct testing of symbiotic properties and found some of the mutants were temperature-sensitive, i.e. they were ineffective at 26° C but effective at 13° C. By use of temperature-shift experiments, such mutants should be useful for studying gene expression during the course of nodule development. However, the actual target site of the mutation in the strains is likely to be difficult to detect.

Mutants of R. trifolii able to synthesize nitrogenase outside the symbiotic state were isolated by selection for methionine sulphoximine resistance (O'Gara and Shanmugam, 1977). The mutant strains were slow-growing, were unable to grow on ribitol or lactose, and had lost the ability to form effective nodules on clover. However, the strains

were able to nodulate soybean effectively (O'Gara and Shanmugam, 1978).

Prior to the commencement of this study, mutants of Rhizobium with specific lesions had not been used to investigate the involvement of the bacteroid in the functioning of the mature nodule.

1.9 Genetic exchange in Rhizobium

Rhizobium strains show wide variability, not only in the ability to form functional nodules with various legumes, but also in other factors of vital agronomic importance. Examples include the ability to persist in the soil in the absence of the host legume ('saprophytic competence'; Chatel et al., 1968) and to compete with other rhizobia in nodule formation (Fred et al., 1932; Hardy and Gibson, 1977; Vincent, 1977). The latter point is especially important if inoculant rhizobia are to be used in areas containing large numbers of indigenous rhizobia. Thus, the development of a system of genetic exchange in Rhizobium is important not only for genetic analysis of the genus, but also to enable the wide variability found in the genus to be exploited for the development of superior inoculant strains.

Many reports of transformation in Rhizobium have appeared in the literature (see Schwinghamer (1977) for review), but in most the controls if used were highly inadequate and thus the occurrence of transformation in the genus remains equivocal. One generalized (Kowalski and Denarie, 1972) and one specialized (Svab et al., 1973, 1978) transducing bacteriophage have been described in R. meliloti. However, a system of conjugation is obviously required if meaningful genetic analysis of the genus is to be done. To this end, Beringer (1974) introduced P-group R factors into R. leguminosarum, but was unable to demonstrate chromosomal recombination although the R factors were readily transferable by conjugation between R. leguminosarum strains. Jacob et al. (1976) inserted segments of R. leguminosarum DNA into RP4 to provide sites of chromosomal homology but the recombinant R factors were as inefficient as RP4 in the transfer of chromosomal markers.

One of the original aims of this study was to look for conjugation in *R. trifolii*, but by the time suitable strains had been constructed, further developments had been reported. Beringer and Hopwood (1976) demonstrated chromosomal recombination (frequency $\sim 10^{-6}$ per marker per recipient) promoted by the R factor R68-45 in *R. leguminosarum*. This R factor was originally derived from R68 on the basis of its ability to promote chromosomal transfer in *Pseudomonas aeruginosa* strain PAO (Haas and Holloway, 1976). It has since been found to differ from R68 by the inclusion of an insertion sequence (Jacob *et al.*, 1977). The R factor R68-45 was used in the conjugation experiments described in this thesis. Since these were largely unsuccessful, the literature concerning conjugation in *Rhizobium* published up to the present is reviewed here.

A circular linkage map of *R. meliloti* was constructed using RP4 (Meade and Signer, 1977) and R68-45 (Kondorosi *et al.*, 1977b) mediated recombination. The linkage map of *R. leguminosarum* was also circular, and the chromosome was transferred and integrated in large segments, enabling the entire chromosome to be covered by seven crosses (Beringer *et al.*, 1978a). Large segments were also transferred and integrated in *R. meliloti* crosses (Meade and Signer, 1977). Johnston and Beringer (1977) found that R68-45 also mediated chromosomal recombination in either direction between *R. leguminosarum* and *R. trifolii* and between *R. leguminosarum* and *R. phaseoli* at similar frequencies to that found in crosses between *R. leguminosarum* strains. This indicated that large segments of DNA from one species were replaceable by DNA from the other species and provided further evidence for the close relatedness of the species (Graham, 1976). Although crosses covering the whole chromosome of *R. leguminosarum* were done, no transfer of host specificity was found (Johnston and Beringer, 1977).

Gene transfer was also detected in crosses between the more distantly related *R. meliloti* and *R. leguminosarum* (Johnston *et al.*, 1978b). Using *R. leguminosarum* as donor, stable haploid recombinants were formed at low frequency, although the *R. leguminosarum* genes

were poorly expressed in R. meliloti. However, in the reverse direction, very few recombinants were found and progeny from these recombinants donated only the selected allele at very high frequency together with the R factor. They were thus R-prime factors. Most of the transferred alleles were poorly expressed in R. leguminosarum.

One of the R-prime factors was able to complement a tryptophan-negative (trp⁻) mutant of P. aeruginosa, showing that rhizobial genes could be expressed in a different genus (Johnston et al., 1978b). This work has recently been extended (Johnston et al., 1978c). Three derivatives of R68-45 that carried different trp genes of R. meliloti were isolated, and their ability to suppress various trp⁻ mutants of P. aeruginosa was tested. Each R68-45-trp plasmid suppressed specific trp alleles of P. aeruginosa and by use of the three R68-45-trp derivatives, all known trp alleles of P. aeruginosa were complemented. This showed that the trp genes of R. meliloti were organized in three clusters and also allowed assignment of allele numbers to each cluster. The trp alleles of R. leguminosarum had previously been mapped to 3 distinct regions of the R. leguminosarum chromosome (Beringer et al., 1978a) and each R68-45-trp derivative from R. meliloti suppressed the trp alleles in one of these regions, showing that the trp genes of the two Rhizobium species were arranged in a similar manner (Johnston et al., 1978c). The R68-45 trp plasmids were unable to suppress any E. coli trp mutant but a derivative of one of the plasmids was isolated which could suppress trpA mutants of E. coli. The failure of the Rhizobium genes to express in E. coli was therefore suggested to be due to a defect in the control of the genes, rather than to the gene products being inactive.

The possibility that genes concerned with symbiotic characters were plasmid-borne was suggested by Higashi (1967) who found that R. phaseoli gained the ability to form infection threads on clover after being cultured in the presence of R. trifolii. Prior culture of the R. trifolii in the presence of acridine orange eliminated its ability to donate the host specificity genes to R. phaseoli. Transconjugant strains capable of

nodulating clover were confirmed to be R. phaseoli by immunological and biochemical tests. This work is unusual amongst most early work purporting to show genetic exchange in Rhizobium in that it used adequate controls. However, the work was not developed further, and is generally regarded with scepticism by present-day workers. There is other indirect genetic evidence for plasmid involvement in infectiveness. Russell and Jones (1973) treated R. trifolii strains with ultra-violet irradiation to give 0.1% survival and found that 86% of the survivors were non-infective. Similarly, Lorkiewicz et al. (1971) found that the majority of auxotrophic mutants of R. trifolii isolated after ultra-violet mutagenesis were non-infective and that prototrophic revertants did not regain infectiveness. The latter workers also found that infectiveness was eliminated by treatment of the R. trifolii strain with acriflavine (Zurkowski et al., 1973). This strain also showed spontaneous loss of infectiveness. Such instability of infectiveness has been observed in a number of rhizobial strains (Labandera and Vincent, 1975).

Biophysical evidence suggests that many rhizobial strains harbour large plasmids (Sutton, 1974; Klein et al., 1975; Tshitinge et al., 1975; Zurkowski and Lorkiewicz, 1976; Nuti et al., 1977), but to date no correlation of genetic and biophysical evidence has been reported. However, very recently Johnston et al. (1978a) demonstrated high frequency transfer of genes specifying nodulating ability from an R. leguminosarum strain to other strains of the same species and to other species (R. trifolii, R. phaseoli, cowpea rhizobia). No chromosomal genes were transferred and thus the genes for nodulating ability were inferred to be on a transmissable plasmid. The R. leguminosarum strain used contained 3 large plasmids (mol. wt. 100 M dal). Further results are awaited.

The interest in plasmid involvement in symbiotic characters of Rhizobium was stimulated by the finding that a large plasmid was required for tumour induction by Agrobacterium tumefaciens (van Larebeke et al., 1974, 1975). This plasmid was transferred to

R. trifolii and the resultant strain was able to both form tumours and nodulate red clover (*Trifolium pratense*). However, it had lost the ability to nodulate *T. parviflorum* and its large plasmid (mol. wt. 250 M dal) was also lost. A plasmid the size of the tumourigenic plasmid was gained (Hooykaas *et al.*, 1977).

Dunican *et al.* (1976) suggested that the genes required for nitrogen fixation (*nif*) in *R. trifolii* were specified by a plasmid which was transferable to *Klebsiella aerogenes*. However, Skotnicki and Rolfe (1978) examined the strain of *R. trifolii* used (TIK) and found it unusual in several respects. The strain was able to transfer *nif* genes to *E. coli* but genes corresponding to the *gal-chlA* region of the *E. coli* chromosome were transferred together with the *nif* genes, suggesting that these genes were picked up from a bacterium with the same gene order as *E. coli* or *Klebsiella*. Strain TIK nodulated clover but also formed crown-galls on other plants and was more similar to *Agrobacterium* than *Rhizobium* in many respects. Thus strain TIK was concluded to be a very unusual bacterium.

To summarize, genetic studies on *Rhizobium* have advanced rapidly in the past few years, and the genetic basis of infectiveness and host-specificity should soon be understood.

A disadvantage of working with *R. trifolii* is the small size of nodules formed on clover, making it impractical to prepare bacteroid extracts for biochemical experiments. However, pea nodules are much larger and bacteroid extracts can be relatively easily prepared. Therefore construction of *R. trifolii* strains able to nodulate peas (Johnston *et al.*, 1978a) will facilitate studies on *R. trifolii* bacteroids.

1.10 Aims of this study

The aim of this study was to investigate the *Rhizobium*-legume symbiosis by genetic analysis of the microsymbiont. Two approaches were to be used. One was to develop a system of conjugation in *R. trifolii* which could be used to analyse the genes involved in the symbiosis. This approach necessitated the isolation of auxotrophic and antibiotic-resistant mutants

and the symbiotic properties of these were to be checked in case they provided an insight into processes important in the symbiosis.

The second approach was to isolate mutants of R. trifolii which were defective in pathways which might be important for nodule function. Two of the key processes in the mature nodule are energy production from carbohydrates and assimilation of ammonia, the product of nitrogenase. The isolation of rhizobial mutants blocked in such pathways was to be attempted so that the involvement of the bacteroid in these processes could be determined. Characterization of such mutants is easier if the pathways involved are known, but such was the ignorance of the basic physiology of rhizobia that this was not the case. Therefore a combined biochemical and genetic approach was to be used in order to delineate the physiologically-important pathways in the free-living rhizobia. Nothing was known about the regulation of carbohydrate or nitrogen metabolism in Rhizobium and it was hoped to use the mutants isolated to study this. Knowledge of the regulatory mechanisms that exist in free-living rhizobia might provide insight into the manner in which metabolism is regulated in bacteroids.

As should be apparent from the literature review, research into areas related to biological nitrogen fixation is progressing rapidly and the emphasis placed on the various aims of this study had to be modified in conjunction with time lost and success gained in order to do original research. Thus the analysis of R. trifolii mutants defective in carbohydrate metabolism forms the larger and more informative part of this thesis.

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacteria

Rhizobium trifolii NZP7 was obtained from R.M. Greenwood, Applied Biochemistry Division, D.S.I.R., Palmerston North, New Zealand, and was relabelled strain 7000 for this work. The majority of the work described in this thesis was done with this strain or derivatives of it. Details of other bacterial strains used are listed in Table 4.

2.2 Media

Defined medium (RDM) contained per litre of distilled water: KH_2PO_4, 1.0 g; K_2HPO_4, 1.0 g; $MgSO_4.7H_2O$, 0.25 g; NaCl, 0.2 g; and $CaCl_2.2H_2O$, 0.02 g. RDM was normally supplemented with trace elements and vitamins as described by Brown and Dilworth (1975) except for biotin which was added to 20 $\mu g\ l^{-1}$. Ammonium chloride (2 mM final concentration) was routinely used as the nitrogen-source except for experiments on nitrogen source utilization (Chapter 5). The nitrogen sources used for these experiments are described in the relevant sections. The phosphates were autoclaved separately and added aseptically to cooled media. Vitamins and NH_4Cl were omitted for RDM nitrogen-free salts medium. Carbon sources were either filter-sterilized (growth rate experiments) or autoclaved separately (other experiments), except for lactulose and raffinose which were always filter-sterilized. The carbon sources were added to a final concentration of 0.4% (w/v) unless otherwise stated. Amino-acids were filter-sterilized and were added, where necessary to supplement the growth of auxotrophs, to a final concentration of 100 $\mu g\ ml^{-1}$. The stock solutions of glutamine were freshly prepared but the stock solutions of other amino-acids were used for up to 6 months. The complex medium used was glucose-salts-yeast extract-casein hydrolyzate (GSYC) (Schwinghamer, 1960), except for a few experiments in which yeast extract agar containing mannitol (YMA) (Vincent, 1970) or glucose (YGA) was used.

Table 4 Bacteria

Strain	Species/Characteristics	Source
W19	*Rhizobium trifolii*	Dr. J.F. Lowe College of Agriculture Edinburgh
P2		
IDL		
FA6		
SU297		Dr. Y. Barnet University of Sydney Australia
SU298		
CR5		New Zealand (C.W.R)
WU1		Dr. M.J. Dilworth University of Western Australia, Perth
VW1-VW40		Isolates from clover nodules obtained in Warwickshire (Mrs. V. Waddel)
301	*R.leguminosarum*	Dr. J.E. Beringer John Innes Institute Norwich
1036		Rothamsted culture collection
6693	*R.meliloti rif* (R68.45)	Dr. A.W.B. Johnston John Innes Institute Norwich
1230	*Escherichia coli* K12 *pro met* (R68.45)	Dr. J.E. Beringer
C603	*E. coli* C *arg his str*	Laboratory stock
MK53	*Klebsiella aerogenes hut* C	Dr. J.E. Brenchley University of Pennsylvania, U.S.A.
CI-one A	*K. pneumoniae*	Laboratory stock

RDM was solidified with Difco Noble agar (1.5 w/v) unless otherwise stated, and GSYC, YMA and YGA with Difco Bacto agar (1.5% w/v). Nutrient agar was prepared from dehydrated material (Oxoid Ltd.). Cycloheximide (10 μg ml^{-1}) was added to solid media when problems with fungal contamination were experienced.

When antibiotics were included in solid media they were added aseptically to the molten agar just prior to pouring into petri dishes. The antibiotics used were dissolved in distilled water and filter-sterilized, except for tetracycline and rifampicin which were dissolved in methanol immediately before use.

2.3 Maintenance and growth of bacteria

Cultures were maintained at -20° C on glass-beads in RDM containing 20% (v/v) glycerol in McCartney bottles. Strains in routine use were subcultured by streaking single colonies on RDM plates every week. All plates and broths were incubated at 28° C. Small-scale cultures were grown in 10 ml broths contained in McCartney bottles. Larger scale cultures were grown in Erlenmeyer flasks filled to half-capacity with medium. The liquid cultures were incubated at 28° C in a Gallenkamp cooled orbitol incubator (Model INR 250) set at a shaking speed of 100 rpm.

2.4 Mutageneses and the isolation of mutant strains

The following procedure was developed for R. trifolii 7000 and its derivatives. Its development is described in Section 3.2.2. Bacteria were grown to a density of approx. 10^8 cells ml^{-1} in the appropriate non-selective medium at which stage 200 μg N-methyl-N'-nitro-N-nitrosoguanidine (NTG) ml^{-1} were added. After 20 min, bacteria were sedimented (6000 g, 10 min), washed twice in the non-selective medium, allowed to grow overnight in the same medium and then either plated out directly or subjected to enrichment. For enrichment, the overnight culture was washed

and diluted to 10^5-10^6 bacteria ml^{-1} in selective medium and allowed to grow for 12 h before ampicillin (25 $\mu g\ ml^{-1}$) was added. After a further 24 h, the bacteria were washed, resuspended in the original non-selective medium and allowed to grow until the culture became turbid. This enrichment cycle was usually repeated.

Enriched cultures were plated for single colonies on selective plates (solidified with Difco Bacto agar). The selective plates contained RDM-mannitol if amino-acid, purine and pyrimidine auxotrophs were to be isolated or RDM containing the selective sugar as sole carbon source if carbohydrate-negative mutants were to be isolated. After 4-16 days, micro-colonies were picked with toothpicks on to media suitable for the identification of mutants. Multiple auxotrophs were isolated by sequential mutageneses.

The requirements of the auxotrophic mutants were determined using the pool-plate method of Holliday (1956). The purine-negative auxotrophs were further characterized by testing for growth stimulation by intermediates of the purine biosynthetic pathway (see Stouthamer et al., 1965; Mohapatra and Kloos, 1975) and by testing for diazotizable amines in the culture supernatants. The latter was done by the Bratton-Marshall test described by Flaks and Lukens (1963). To prepare culture supernatants, bacteria were grown in RDM-glucose broth, containing 5 $\mu g\ ml^{-1}$ adenine, for 6 days. The bacteria were then sedimented and the supernatant fluid used for the Bratton-Marshall test. A portion of each supernatant fluid was acid-hydrolyzed (10 min, 100° C, 0.2N HCl) before doing the test.

Antibiotic-resistant mutants were isolated by spreading 0.1 ml portions of a washed suspension of bacteria ($10^9\ ml^{-1}$) on to fresh GSYC plates containing the relevant antibiotic. The suspension was prepared from bacteria in the late-exponential phase of growth in GSYC broth.

2.5 Genetical procedures

Crosses between *R. trifolii* strains, between *R. leguminosarum* and *R. trifolii*, between *R. meliloti* and *R. trifolii* and between *E. coli* and *R. trifolii* were done as described for *R. leguminosarum* crosses by Jacob *et al.* (1976). Cultures (0.5 ml) of donor and recipient bacteria in late exponential phase were mixed in 10 ml sterile water and filtered on to a Millipore membrane filter (0.45 μm pore size, 47 mm diameter). The membrane was then placed on a GSYC plate and incubated at 28° C for 1-2 days. The bacteria on the membrane were then resuspended in 5 ml RDM-nitrogen-free salts medium and a series of dilutions in the same medium were plated on to suitable selective media. Occasionally when only transfer of the R-factor from one strain to another was desired, a loopful of the donor and of the recipient strain were mixed on the surface of a GSYC plate and the next day a loopful of the resultant growth was streaked on to selective medium.

The level and types of resistance expressed by the appropriate strains were tested by placing suitable Oxoid Multodisks on a freshly spread bacterial lawns on GSYC plates.

2.6 Preparation of cell-free extracts

Initial attempts to grow 500 ml broth cultures using 10 ml late-exponential broth inocula gave unreliable growth. Therefore, the following procedure was developed to ensure that cultures were in exponential phase and ready for use when required (usually 24 h after inoculation). One litre Erlenmeyer flasks containing 500 ml of medium were inoculated with bacteria washed off fresh plates containing an appropriate medium and streaked 4 days previously. Enough inoculum was added to give approximately 10^7 bacteria ml^{-1}. Just before harvesting, cultures were checked for contamination by streaking on nutrient agar plates (*Rhizobium trifolii* does not grow on nutrient agar), and on appropriate RDM plates for phenotype confirmation.

Bacteria were harvested while they were in the exponential phase of growth (A_{540} of 0.1-0.5), washed twice in 20 mM potassium phosphate buffer (pH 7.6) at room temperature, washed and resuspended once in cold (4° C) 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulphonate (HEPES) buffer (pH 7.6) containing 1 mM dithioerythritol and passed once through a French pressure cell at 137 MPa. The extract was then centrifuged at 38000 g for 1 h at 4° C and the supernatant fluid was used for assays. If the particulate fraction was to be assayed, a preliminary centrifugation at 6000 g for 10 min was incorporated to remove whole cells, and if NADH-linked enzymes were to be assayed, the time of centrifugation at 38000 g was extended to 2 h.

2.7 General spectrophotometric procedures

The assays for glutamate dehydrogenase and glutamate synthase (GOGAT) were done with a Unicam SP1800 UV recording spectrophotometer. All other spectrophotometry was done using a Cecil CE202 spectrophotometer fitted with a cuvette carriage which could be temperature-controlled by means of a water circulator, and which was coupled to a Servoscribe Is recorder

2.8 Determination of growth

The growth of bacteria on solid media was determined by one of two methods. Either bacteria from a single colony were streaked for single colonies on to the appropriate medium or a culture of bacteria was diluted and spread for single colonies. The amount of growth was recorded after incubation of the plates at 28° C for the specified time.

Doubling times in liquid media were measured on cultures growing at 28° C with gentle shaking in 250 ml Erlenmeyer flasks containing 100 ml of appropriate medium. The inocula were prepared from streaked RDM plates containing inositol and the required amino-acid(s), and the flasks were inoculated to give

approximately 10^6 bacteria ml^{-1}. The growth was monitored by measuring the absorbance of 1 ml samples of the cultures at 540 nm from 16 to 96 h after inoculation. Standard curves of A_{540} versus dry weight and A_{540} versus viable cell number were constructed.

2.9 Enzyme assays

2.9.1 General procedures.- In most cases, enzymes were assayed according to published methods and no attempt was made to optimize reagent concentrations, etc. All enzymes were assayed at 28° C. All pyridine nucleotide-linked enzymes were assayed in a final volume of 1.0 ml by following the change in absorbance at 340 nm.

2.9.2 Enzymes of nitrogen assimilation

2.9.2.1 Alanine dehydrogenase.- (L-alanine:NAD oxidoreductase (deaminating): EC 1.4.1.1) was assayed according to Rowell and Stewart (1976). The reaction mixture contained 100 µmol Tris HCl (pH 8.0), 0.1 µmol NAD(P)H, 100 µmol NH_4Cl, and extract. The reaction was started by the addition of 1 µmol sodium pyruvate.

2.9.2.2 Glutamate dehydrogenase.- (GDH, L-glutamate: NAD(P) oxidoreductase (deaminating); EC 1.4.1.3) was assayed according to Cole et al. (1974). The reaction mixture contained 100 µmol HEPES (pH 7.6), 50 µmol NH_4Cl, 0.18 µmol NAD(P)H and extract. The reaction was started by the addition of 5 µmol 2-oxoglutarate (pH 7.0). Stock solutions of NADPH and 2-oxoglutarate were stored frozen and were discarded after two weeks.

2.9.2.3 Glutamate synthase.- (Glutamine (amide):2-oxoglutarate aminotransferase oxidoreductase ($NADP^+$)) (GOGAT) was assayed according to Cole et al. (1974). The reaction mixture contained 100 µmol HEPES (pH 7.6), 3.3 µmol glutamine, 0.18 µmol NADPH and extract. The reaction was started by the addition of 5 µmol 2-oxoglutarate. Addition of glutamine last gave similar results.

2.9.2.4 Glutamine synthetase .- (GS; L-glutamine:ammonia ligase (ADP-forming): EC 6.3.1.2) was assayed by both the transferase and the biosynthetic reactions. The reaction mixture for the transferase assay was that of Shapiro and Stadtman (1970) as modified by Foor et al. (1975), i.e. the reaction mixtures were prepared exactly as described by Shapiro and Stadtman (1970) except that the pH of the imidazole buffer was 7.15. The total enzyme activity was measured in the presence of 0.3 mM $MnCl_2$ and that of the 'unadenylylated' enzyme in the presence of 0.3 mM $MnCl_2$ plus 60 mM $MgCl_2$. Control tubes minus ADP and arsenate were used to correct for glutaminase activity.

The biosynthetic enzyme assay used was that of Elliot (1955) except that 0.1 M imidazole (pH 7.15) was used as buffer.

2.9.2.5 Histidase.- (histidine ammonia-lyase; EC 4.3.1.3) was assayed as described by Magasanik et al. (1971) for the enzyme in cell-free extracts.

2.9.3 Enzymes of carbohydrate metabolism

2.9.3.1 Glucokinase .- (ATP:D-glucose 6-phosphotransferase; EC 2.7.1.2) was assayed according to Anderson and Kamal (1966). The reaction mixture contained 100 µmol HEPES (pH 7.6), 10 µmol $MgCl_2$, 20 µmol D-glucose, 1 µmol $NADP^+$, glucose-6-phosphate dehydrogenase (0.4 units) and extract. The reaction was started after 2 min by the addition of 5 µmol ATP.

2.9.3.2 Glucose dehydrogenase .- (Glucose:(acceptor) oxidoreductase (EC 1.1.99a); β-D-glucose: NAD(P) oxidoreductase (EC 1.1.1.47)). Various assays were used in efforts to find activity of either of these enzymes. Glucose dehydrogenase (EC 1.1.1.99a) was assayed both according to Hauge (1966) and according to Ng and Dawes (1973). The latter assay was modified by the addition of phenazine methosulphate (2 mM) (Whiting et al., 1976a). The mixtures used for the glucose dehydrogenase (EC 1.1.47) assays were as described by Sadoff (1966) and Keele et al. (1970). Both NAD and NADP were tested as co-factor.

2.9.3.3 Gluconate dehydrogenase .-(D-gluconate:(acceptor) oxidoreductase, EC 1.1.99.3) was assayed according to the methods of Ng and Dawes (1973) and Keele et al. (1970).

2.9.3.4 Gluconokinase.- (ATP:D-gluconate 6-phosphotransferase; EC 2.7.1.12) was assayed according to the method of Ng and Dawes (1973).

2.9.3.5 Fructokinase (ATP:D-fructose 6-phosphotransferase; EC 2.7.1.4) was assayed in a similar manner to glucokinase except that fructose replaced glucose in the assay mixture.

2.9.3.6 Glucose phosphate isomerase.- (D-glucose-6-phosphate ketol-isomerase: EC 5.3.1.9) was assayed according to Noltman (1966). The reaction mixture contained 100 µmol HEPES (pH 7.6), 0.5 µmol $NADP^+$, glucose-6-phosphate dehydrogenase (~ 2 units) and enzyme. The reaction was started by the addition of 10 µmol fructose-6-phosphate.

2.9.3.7 Glucose-6-phosphate dehydrogenase.- (G6PDH; D-glucose-6-phosphate : NADP oxidoreductase; EC 1.1.1.49) was assayed according to Kuby and Noltman (1966). The reaction mixture contained 100 µmol HEPES (pH 7.6), 10 µmol $MgCl_2$, 1 µmol NADP and extract. The reaction was started by the addition of 1 µmol glucose 6-phosphate.

2.9.3.8 Phosphogluconate dehydrogenase.- (6PGDH; 6-phospho-D-gluconate: $NADP^+$ oxidoreductase (decarboxylating); EC 1.1.1.44) was assayed in a similar manner to G6PDH except that 6-phospho-gluconate replaced glucose 6-phosphate in the reaction mixture.

2.9.3.9 Fructose diphosphate aldolase.- (FDA; fructose-1,6-diphosphate-D-glyceraldehyde-3-phosphate-lyase; EC 4.1.2.13) was assayed by all the methods described by Rutter et al. (1966).

2.9.3.10 Phosphofructokinase.- (PFK; ATP:D-fructose-6-phosphate-1-phosphotransferase; EC 2.7.1.11) was initially assayed according to Ling et al. (1966). Since no activity of this enzyme was found, various other conditions were also tested (see Section 6.2.1).

2.9.3.11 Galactose dehydrogenase.- The reaction mixture for galactose dehydrogenase contained 100 µmol HEPES (pH 7.6), 3 µmol $MgCl_2$, 1 µmol $NADP^+$ and extract. The reaction was

started by the addition of 2 μmol galactose. The NAD^+-dependent galactose dehydrogenase (EC 1.1.1.48) was assayed in a similar manner except that 50 μmol sodium carbonate/bicarbonate buffer (pH 9.7) was used in place of HEPES, and NAD^+ was used in place of $NADP^+$.

2.9.3.12 Polyol dehydrogenases were assayed according to Martinez-de Drets and Arias (1970). The reaction mixture contained 50 μmol sodium carbonate/bicarbonate buffer (pH 9.7), 0.6 μmol NAD^+ and extract. The reaction was started by the addition of 20 μmol of the relevant polyol.

2.9.3.13 Entner-Doudoroff pathway enzymes.- The activity of the ED pathway ('ED enzyme') was determined by following the production of pyruvate from 6-phosphogluconate (6PG). The reaction mixture contained 20 μmol HEPES (pH 7.6), 5 μmol 6PG, 4 μmol $MgCl_2$, 3 μmol DTE and extract (approximately 1 mg protein) in a final volume of 1 ml. Assays were run at 30° C for the specified time (usually 10 min) and the pyruvate formed was determined by the method described by Keele et al. (1969). No attempt was made to separate phosphogluconate dehydrogenase (6-phospho-D-gluconate hydro-lyase; EC 4.2.1.12) and 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (6-phospho-2-keto-3-deoxy-D-gluconate D-glyceraldehyde-3-phosphate lyase; EC 4.1.2.14) activities because of the unavailability of KDPG.

2.9.3.14 (β-galactosidase.- β-D-galactoside galactohydrolyase; EC 3.2.1.23) was assayed by following the hydrolysis of 3.3 μmol of either orthonitrophenyl-β-D-galactopyranoside (ONPG) or para-nitrophenyl-β-D-galactopyranoside (PNPG) in the presence of 100 μmol HEPES (pH 7.6) and extract in a final volume of 1 ml. The increase in absorbance at 420 nm was monitored and the reaction rate was constant for at least 20 min. The addition of Mn^{2+}, Mg^{2+} or Na^+ ions had no effect on the level of activity obtained. The assay was done at 30° C although the activity of

the enzyme was increased approximately fifty per cent at 37° C. The molar extinction coefficient of ortho- and paranitrophenol at 420 nm and in a 1 cm lightpath was taken to be 4700.

2.9.3.15 Isocitrate dehydrogenase.; (threo-D-isocitrate: NADP oxidoreductase (decarboxylating); EC 1.1.1.42). The reaction mixture contained 20 µmol HEPES (pH 7.6), 2 µmol $MnSO_4$, 1 µmol DTE, 1 µmol $NADP^+$ and extract. The reaction was started by the addition of 8 µmol DL-isocitrate.

2.9.3.16 Malate dehydrogenase.- (L-malate: NAD oxidoreductase; EC 1.1.1.37) was assayed as described by Englard and Siegel (1969).

2.9.4 Anaplerotic enzymes

2.9.4.1 Carboxylases.- The carboxylase reaction mixtures described below were incubated for 10 min before the reactions were started by the addition of either pyruvate or PEP. For avidin treatment, 50 µg avidin were added to each reaction mixture before pre-incubation. Details of other controls are given in Chapter 6, Table 28. After 10 min, the reactions were stopped by the addition of 200 µl of 12 M formic acid and 200 µl of the mixture were then transferred to a scintillation vial and dried at 80° C for at least 3 h. The residue was then suspended in 1 ml H_2O, 10 ml of scintillation fluid were then added and the radioactivity was determined. Counting efficiency was calculated to be 63%.

2.9.4.1.1 Pyruvate carboxylase.- (pyruvate:carbon dioxide ligase) (ADP); EC 6.4.1.1). The assay was modified from that described by Phibbs et al. (1974). The reaction mixture contained 20 µmol Tris HCl (pH 7.8), 0.5 µmol sodium pyruvate, 0.8 µmol ATP, 4 µmol $MgCl_2$, 0.034 µmol acetyl CoA, 0.63 µmol NADH, 2 µmol DTE, 12.5 µmol ^{14}C-labelled $NaHCO_3$ (1.6 µCi µmol^{-1}) and 200 µl extract in a final volume of 0.5 ml.

2.9.4.1.2 Phosphoenolpyruvate (PEP) carboxylase.- (pyrophosphate: oxaloacetate carboxylase (phosphorylating); EC 4.1.1.38). To assay PEP carboxylase, ATP was omitted from the pyruvate carboxylase reaction mixture described above and the pyruvate was replaced by 0.5 µmol PEP.

2.9.4.1.3 PEP carboxykinase. - (NTP: oxaloacetate carboxy-lyase (transphosphorylating); EC 4.1.1.32). To assay PEP carboxykinase, 0.2 µmol ADP were added to the PEP carboxylase reaction mixture described above.

2.9.4.2 Malic enzyme. - (L-malate: NADP oxidoreductase (decarboxylating), EC 1.1.1.40) was assayed according to Hsu and Lardy (1969).

2.9.4.3 Isocitrate lyase. - (threo-D-isocitrate glyoxylate-lyase; EC 4.1.3.1) was assayed according to Johnson et al. (1966).

2.9.4.4 Malate synthetase. - (L-malate glyoxylate-lyase (CoA-acetylating); EC 4.1.3.2) was also assayed according to Johnson et al. (1966) except that acetyl CoA was purchased from commercial sources (Sigma).

2.9.5 NADH oxidase. - The assay mixture for NADH oxidase contained 100 µmol HEPES (pH 7.6) and 2 µmol NADH. The reaction was started by the addition of extract.

2.9.6 Specific activities of enzymes in crude extracts were expressed as nanomoles (nmol) of substrate transformed min^{-1} (mg protein)$^{-1}$. A value of 5.60×10^3 was found for the molar extinction coefficient of NAD(P)H using the Cecil CE202 spectrophotometer, and this value was taken when calculating the specific activities of pyridine nucleotide-linked enzymes assayed in this spectrophotometer. The published value of 6.22×10^3 was used for assays done in the Unicam SP1800 spectrophotometer.

2.10 Protein was determined by the biuret method (Herbert et al., 1971), using dried crystalline bovine serum albumin as standard.

2.11 Uptake of radioactive sugars

The procedure used was modified from that described by Kornberg and Riordin (1976). Bacteria grown in RDM with the appropriate carbon source were harvested during the exponential

growth phase (A_{540} of 0.1-0.4), washed once at room temperature with RDM nitrogen-free salts medium, resuspended in the same medium to an absorbance (540 nm) of 1.00 (10^9 bacteria ml^{-1}; 0.65 mg dry weight ml^{-1}) and used immediately. A sample (1.8 ml) of this suspension was shaken at 28° C in a 25 ml Erlenmeyer flask for 10 min before the addition of 0.2 ml ^{14}C-labelled sugar to give a final concentration of 1 mM (1.25 µCi μmol^{-1}). Samples were taken rapidly at specified time intervals, filtered with suction through Millipore filters (0.45 µm pore size, 25 mm diameter) and washed twice with 2 ml of RDM nitrogen-free salts medium. Filters were then transferred to scintillation vials, dried (60° C, 30 min), cooled and counted in 10 ml of scintillation fluid.

2.12 Measurement of radioactivity was done in a Packard Tri-carb liquid scintillation spectrophotometer. The scintillation fluid used had the following composition: toluene, 1 l; Triton X-100, 0.5 l; 2,5-diphenyloxazole (PPO), 6 g; 1,4-di-2-(5-phenyloxazolyl)-benzene (POPOP), 75 mg.

2.13 Nodulation tests were done using Gibson's partly enclosed seedling method and Jensen's seedling solution (Vincent, 1970). The test cultivar was red clover (Trifolium pratense) var. Attaswede.

Seeds were surface-sterilized by immersing in ethanol for 30 sec followed by 3 min in 0.2% acidified $HgCl_2$ and six washes in sterile distilled water. The seeds were then placed on to the surface of water agar (0.6% w/v) plates (20 per plate) which were inverted and left at 4° C for at least two days. When required, the seeds were germinated by placing the plates at 20° C in the dark for 30 h. Uniformly-germinated seedlings (radicle approx. 10 mm long) were then planted in test-tubes prepared as described below.

13 ml of Jensen's seedling agar (Vincent, 1970) were added to 150 mm x 20 mm test-tubes which were then capped with a 5 cm square of double-thickness aluminium foil (Alcan) which was

secured with a nylon strap. The tubes were then autoclaved and set at a slope so that the agar just reached the foil cap. To plant the seedling, a small hole was made in the cap above the edge of the agar slope and the radicle was inserted so that it lay along the surface of the slope. The tubes were then placed in a container which was placed in a plant-growth room.

To prepare a suitable container, the lids were cut off a test-tube carton (supplied with 200 mm x 30 mm tubes) and the top of the carton was covered with aluminium foil (8-fold thickness). The foil was pierced above each test-tube partition, and a seedling test-tube was forced into each partition in such a way that the test-tube was rested on the foil surface by the strap around the top of the tube. Seventy-two tubes were placed in each carton, and the carton was placed in a larger open-topped carton which was enclosed by a transparent plastic bag. A beaker of water was included in the carton to provide a humid atmosphere.

After 3 days, the seedling carton was removed from the 'humidity chamber' and the seedling tubes were watered and inoculated. A hole was made opposite the seedling and seedling solution (quarter-strength, Vincent, 1970) was added to approximately 10 mm from the top of the tube. A few drops of inoculum (10^9 bacteria ml^{-1}) were then added and the hole was sealed with a sterile rubber bung (5 mm). All operations were done in a laminar-flow hood and uninoculated controls were included. At least two seedlings were inoculated with each strain tested. The inocula were prepared by washing the growth off RDM plates with seedling solution (quarter strength).

After inoculation, the carton containing the tubes was placed in a plant-growth room at 20° C under 10000 lux and an $8\frac{1}{2}$ h or 16 h day-length. The tubes were topped up with diluted seedling solution whenever necessary. The use of Gibson's partly enclosed seedling method allowed onset of nodulation and plant response to be monitored. Placement of the tubes in the carton gave equal spacing of tubes, and allowed root development to take place in the dark while allowing easy access for monitoring nodulation.

2.14 Nitrogen fixation was estimated by the acetylene reduction method (Bergersen, 1970). Test-plants were removed from the growth-room 2 h into the light cycle, and entire root systems were cut off the plate and transferred to McCartney bottles containing 0.5 ml H_2O. The bottles were capped with rubber septa ('Suba Seal', Wm. Freeman & Co., Barnsley, Yorkshire) and placed in a water bath at 25° C. Air (2.5 ml) was removed and 2.5 ml acetylene added. Gas samples (0.4 ml) were withdrawn after 5 min and 35 min and analyzed using a Pye Unicam Series 104 chromatograph with a flame ionization detector, a 1.6 m x 6 mm column of Porapak R at 60° C and a nitrogen carrier gas flow of 40 ml min^{-1}. The amount of ethylene formed was calculated from a standard curve constructed using suitably diluted ethylene obtained from a cylinder. The height of the acetylene peak was monitored as an internal standard.

2.15 Isolation of strains from nodules

Nodules were surface-sterilized by the method described for surface-sterilizing seeds. Sterile nodules were crushed in a drop of sterile water using a sterile spatula, and the milky suspension obtained was streaked out on to GSYC and on to media suitable for demonstration of the phenotype of the strain.

2.16 Chemicals

Biochemicals and enzymes used in enzyme assays were the best grade available and were mainly from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Sugars used as carbon sources were either 'Analar' grade and purchased from B.D.H. Chemicals, Poole, Dorset, U.K. or Fisons Scientific Apparatus, Loughborough, Leicestershire, or were the best grade available from Sigma. Yeast extract used in complex media was from Difco Laboratories, West Molesey, Surrey, and the casein hydrolyzate used in GSYC was from B.D.H. Chemicals.

The following radioisotopic material was purchased from the Radiochemical Centre, Amersham, Buckinghamshire, U.K. and was used without further purification: D-[U-^{14}C]glucose (292 mCi mmol^{-1}), D-[U-^{14}C]fructose (303 mCi mmol^{-1}), D-[1-^{14}C] mannitol (60 mCi mmol^{-1}), [D-glucose-1-^{14}C] lactose (20 mCi mmol^{-1}), D-[1-^{14}C]dulcitol (7.1 mCi mmol^{-1} and sodium [^{14}C] bicarbonate (40 mCi mmol^{-1}). The D-[1-^{14}C] dulcitol was a special purchase (non-catalogue item) and was estimated to be 98% pure in 1972, a loss of < 1% since the same batch was previously assayed in 1969. D-[1-^{14}C]ribitol (1.88 mCi mmol^{-1}) was purchased from New England Nuclear, Winchester, Hampshire.

2.17 Gases

Acetylene and ethylene were obtained from British Oxygen Co., London, U.K. Over a period of 6 months when cylinder acetylene was unavailable, acetylene was generated by the action of water on calcium carbide, using equipment similar to that described by Postgate (1972).

CHAPTER 3

ISOLATION AND SYMBIOTIC PROPERTIES OF AUXOTROPHIC AND ANTIBIOTIC-RESISTANT MUTANTS

3.1 Introduction

The aim of the work described in this chapter was to isolate mutants of R. trifolii for use in conjugation experiments. Therefore, multiply-marked strains of R. trifolii were to be constructed. The symbiotic properties of the auxotrophic and antibiotic-resistant mutants were to be checked in case they provided insight into processes important in nodule formation and function (see Section 1.8).

3.2 Results

3.2.1 Studies on R. trifolii strain W19

Initial experiments were done with R. trifolii strain W19 which was selected from laboratory stocks because of its low slime production and good growth rate on GSYC. Killing curves for NTG (200 $\mu g\ ml^{-1}$ final concentration) were done using cultures of strain W19 either growing in GSYC or suspended in Tris-maleic buffer (pH, 6.0; 0.04 M). Very similar curves were obtained under both conditions and so all further NTG treatments were done in GSYC. In initial experiments, cultures were mutagenized to give 40% survival (7 min), washed twice in GSYC and allowed to segregate overnight in GSYC. Cultures were then diluted and spread on GSYC plates and the resultant single colonies were tested for possible auxotrophic mutants by picking on to RDM-glucose plates solidified with Difco-Bacto agar. A similar procedure gave 1% auxotrophs. from R. leguminosarum (Beringer, 1973). However, only 4 auxotrophic mutants of strain W19 were found from more than 2500 colonies tested in 3 mutagenesis experiments.

When testing the growth of presumptive mutants on pool plates (Holliday, 1956), it was noticed that the wild-type W19 formed a poor lawn on control RDM-glucose plates but was stimulated by several of the pools. This result was surprising because strain W19 grew well when spread for single colonies on to the same medium. The possibility that wild-type strain W19 had an auxotrophic requirement

which was satisfied by impurities in the Difco Bacto agar was tested by spreading duluted cultures for single colonies in RDM-glucose solidified with Difco Noble agar. The growth obtained on this medium was compared to that obtained on the same medium solidified with Difco Bacto agar. Colonies obtained on the medium solidified with Difco Bacto agar were easily visible after 4 days and had grown to 1.2 mm by 6 days whereas colonies on the medium containing Difco Noble agar were barely visible after 4 days. Thus strain W19 was able to scavenge essential nutrients from the Difco Bacto agar. Subsequently all defined media were solidified with Difco Noble agar. A similar phenomenon of growth on solid but not in liquid medium had been reported by Beringer (1973) for various R. leguminosarum strains. Strain 301 was obtained from Beringer and was found to exhibit similar properties to strain W19.

Strain W19 was unable to grow on RDM-glucose solidified with Difco Noble agar or in RDM-glucose broth unless the medium was supplemented with one or more of the following: aspartate, glutamate, histidine or proline. The growth properties and enzymatic defects of strain W19 are described in Sections 5.2.1 and 5.2.2. By use of pool-plates supplemented with glutamate, the requirements of 3 of the 4 W19 auxotrophs isolated were identified as adenine or hypoxanthine (pur-1), histidine (his-1) and methionine (met-1). The growth of the fourth auxotroph was not stimulated by any of the pools.

Antibiotic-resistant mutants (resistant to 100 μg rifampicin ml^{-1} (rif); 200 μg streptomycin ml^{-1} (str); 200 μg spectinomycin ml^{-1} (spc); 300 μg erythromycin ml^{-1} (ery))of both parental and auxotrophic strains were isolated in order to construct strains suitable for genetic crosses (see Section 4.2.2). The rif, str and spc mutants arose at a frequency of 10^{-6}-10^{-8} and within 3-4 days incubation had grown to 1 mm diameter on GSYC plates containing the relevant antibiotic. However, ery mutants did not appear on GSYC-erythromycin plates prior to 14 days incubation and appeared spasmodically after this time. The growth rate of purified ery mutants on GSYC in the presence or absence of erythromycin was similar to that of wild-type W19.

However, the long lag phase prior to the initial appearance of the ery mutants may explain the difficulty other workers have had in isolating these mutants (Beringer, personal communication). No mutants (from > 5 x 10^{10} bacteria plated) resistant to tetracycline (10 μg ml^{-1}) or oxytetracycline (10 μg ml^{-1}) were obtained.

It was then found that strain W19 and its mutant derivatives, except W19 pur-1, formed ineffective nodules on red clover (var. Attaswede). Strain W19 pur-1 did not even nodulate red clover. Acetylene-reduction tests confirmed that W19 was ineffective as originally deduced from nodule colour and plant response. Mutants of W19 able to grow on unsupplemented RDM-glucose (see Section 5.2.1) were also ineffective. Since one of the aims of this investigation was to determine the effect of various mutations on symbiotic properties, W19 was considered unsuitable for further work.

3.2.2 Initial studies on R. trifolii 7000

Strain 7000 was selected for further work for the following reasons: (i) it was fully effective on red clover; (ii) it had been maintained in laboratory stocks since 1953 without showing any alteration in symbiotic properties (Greenwood, personal communication), indicating that it was relatively stable; (iii) it produced relatively little slime; (iv) it grew well in GSYC and in unsupplemented RDM-glucose; (v) it was susceptible to many antibiotics, including those useful for mutant enrichment (ampicillin, carbenicillin) and R-factor selection (carbenicillin, kanamycin, tetracycline); (vi) it was susceptible to a range of bacteriophage; (vii) it was susceptible to lysozyme, and hence easily lyzed for DNA isolation. Strain 7000 was the only R. trifolii strain out of eight tested to display this property.

The observation that auxotrophic mutants of W19 grew to visible size on RDM-glucose solidified with Difco Bacto agar led to the development of an auxotroph selection procedure. The bacteria of strain 7000 were mutagenized with NTG to give 40% survival

(200 μg ml^{-1}, 20 min) and grown overnight in GSYC to allow for segregation. The cultures were then diluted in RDM and plated out (~ 100 colonies per plate) for single colonies on RDM-glucose solidified with Difco Bacto agar. Very small colonies ('micro-colonies') which appeared after 4-16 days incubation were picked off the plates on to GSYC and RDM-glucose (Difco Noble agar) plates. In a total of three experiments, 49 of the 800 micro-colonies tested had an auxotrophic requirement. This frequency (6.1%) compared favourably to the frequency previously obtained (0.16%) by testing colonies of W19 picked randomly from GSYC plates.

The growth of strain 7000 in RDM-glucose broths containing ampicillin was then tested. The bacteria from an overnight culture grown in GSYC were washed twice and inoculated (final concentration 10^6 cells ml^{-1}) into a series of broths containing increasing concentrations of ampicillin. No growth was observed in broths containing more than 5 μg ml^{-1} ampicillin, even after 4 days incubation. The frequency of spontaneous mutation to ampicillin resistance was then determined. Mutants resistant to 5 or 10 μg ml^{-1} ampicillin arose at a frequency of 10^{-5} to 10^{-6}, whereas mutants resistant to 25 or 50 μg ml^{-1} ampicillin were approximately 100-fold less frequent. The effect of ampicillin (25 μg ml^{-1}) on the viability of bacteria of strain 7000 growing in RDM-glucose broths was then determined. To do this, bacteria grown overnight in GSYC were washed twice and inoculated into two RDM-glucose broths (final concentration, 10^6 cells ml^{-1}). These cultures were incubated for 12 h, which allowed approximately 3 divisions of the bacteria, and then the ampicillin was added to one of them. After a further 24 h, the viable cell number, as determined by plating on RDM-glucose (Difco Bacto agar) had decreased to approximately 250 ml^{-1} in the ampicillin-treated culture, whereas the control culture contained approximately 10^8 bacteria ml^{-1}. No further decrease in viable cell number in the ampicillin-treated culture was observed upon further incubation of the culture. These experiments suggested that the treatment of

cultures growing in RDM-glucose with ampicillin (25 $\mu g\ ml^{-1}$) for 24 h would constitute an efficient selection procedure for auxotrophic mutants.

The use of ampicillin enrichment did not give a dramatic increase in the yield of auxotrophic mutants. However, since an adequate number of such mutants were obtained from picking micro-colonies, this was not investigated further. Nevertheless, in one experiment using two independent cultures of auxotrophic mutants, it was found that after ampicillin enrichment followed by growth to turbidity in GSYC, over 50% of the colonies obtained on appropriately supplemented RDM-glucose (Difco Bacto agar) plates were micro-colonies. Most of these micro-colonies also grew poorly on GSYC plates and were eventually found to be glucose-negative. The properties of these mutants are described further in Chapters 6 and 7.

An uneven spread of auxotrophs was obtained using the 'micro-colony' method, with the majority of the mutants being purine-dependent (see Table 5). Whether this was a reflection of the method used was not investigated; however, the non-purine-dependent mutants obtained exhibited a tight phenotype in unsupplemented RDM-glucose broths. Of these mutants, two which had a low reversion frequency (strains 7008, trp and 7012, his) were used as parents for a further NTG mutagenesis experiment in order to obtain doubly-marked strains for use in conjugation experiments. Suitably marked strains (Table 5) were found from amongst micro-colonies obtained by plating bacteria after mutagenesis and overnight growth but prior to ampicillin enrichment. As described above, most of the bacteria obtained after ampicillin enrichment in these experiments were glucose-negative.

3.2.3 Biochemical characterization of purine-negative mutants

Since the majority of the auxotrophs isolated were purine-dependent, it was decided to further characterize these mutants. The experiments were done primarily to identify different pur

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Table 5 Auxotrophic mutants of R. trifolii isolated by the 'micro-colony' method

Parent strain	Requirement	No. isolated
7000	adenine or hypoxanthine	17
	guanine	4
	uracil	4
	nicotinic acid	3
	glutamate or glutamate-yielding amino-acids	2
	histidine (his-6)	1
	leucine	2
	methionine	1
	tryptophan (trp-4)	1
	unidentified	15
7008 (trp-4)	adenine	1
	adenine or hypoxanthine	2
	uracil or cytosine	1
	choline	1
	methionine or cysteine	1
	unidentified	6
7012 (his-6)	adenine or hypoxanthine	3
	uracil	1
	unidentified	3

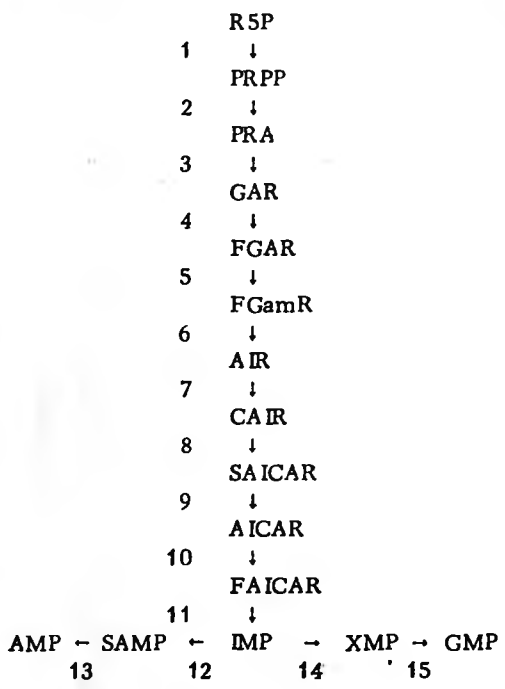
*Requirements were identified using the pool-plates (Holliday, 1956)

alleles that might be useful for genetic mapping and complementation studies. A secondary consequence of the experiments was to provide information on the pathway of biosynthesis and interconversion of purines in R. trifolii.

The pathway of purine biosynthesis found in pigeon liver and in Escherichia coli is outlined in Fig. 2. Although AIR is an intermediate in thiamine biosynthesis, the use of the pool-plates (Holliday, 1956) should have allowed identification of mutants of R. trifolii blocked prior to the formation of this compound since RDM routinely contained thiamine. The growth of the 27 purine-dependent mutants isolated was tested on RDM-glucose plates supplemented with various purine intermediates. The results enabled the mutants to be placed into 5 groups (Table 6). It should be noted that the mutants in Group V were leaky, which may have masked the true effects of supplementation.

Many intermediates in the purine biosynthetic pathway are derivatives of amino-imidazoles, and the amino groups of these compounds can be diazotized. Such diazotized compounds can then be detected colorimetrically by the Bratton-Marshall test (Flaks and Lukens, 1963). After such treatment, AIR gives an absorption maximum at 502 nm and AICAR and SAICAR at 540 nm. The Bratton-Marshall test was done on culture supernatants from all of the mutants as described in Section 2. A portion of each supernatant was acid-hydrolyzed prior to testing, since acid-hydrolysis converts FAICAR to AICAR (Mohapatra and Kloos, 1975) which can then be detected. The mutants in group III accumulated large amounts (up to 5 μmol (ml culture)$^{-1}$) of an intermediate which gave a sharp absorption maximum at 502 nm, characteristic of AIR. These mutants also excreted a brown pigment into the medium in which they were grown. The mutants in group V accumulated small amounts of an intermediate which reacted in the Bratton-Marshall test to give a chromophore with an absorption maximum at 520 nm. However, no intermediates

Figure 2 Pathway of purine biosynthesis (adapted from Stouthamer et al., 1965)



Abbreviations: R5P, ribose 5-phosphate; PRPP, 5-phosphoribosyl-1-pyrophosphate; PRA, 5-phosphoribosylamine; GAR, glycinamide ribonucleotide; FGAR, formyl glycinamide ribonucleotide; FGamR, formyl glycinamidine ribonucleotide; AIR, 5-aminoimidazole ribonucleotide; CAIR, 5-amino-4-imidazole-carboxylic acid ribonucleotide; AICAR, 5-amino-4-imidazole-carboxamide ribonucleotide; SAICAR, 5-amino-4-imidazole-carboxamide ribonucleotide; FAICAR, 5-formamido-4-imidazole-carboxamide ribonucleotide; IMP, inosinic acid; SAMP, adenylsuccinic acid; AMP, adenylic acid; XMP, xanthylic acid; GMP, guanylic acid.

Table 6 Growth of purine-dependent mutants on RDM-glucose plates supplemented with various intermediates of the purine biosynthetic pathway

The mutants were patch-streaked with the blunt end of a toothpick from GSYC plates on to the supplemented RDM-glucose plates

		Supplement*							
Group	Days after streaking	None	AICA	AICAR	hyx	ade	xan	gua	No. of mutants
I	2	-	-	±	+	±	-	-	14
	5	-	±	+++	+++	+++	-	-	
II	2	-	-	-	-	-	±	-	4
	5	-	±	-	-	+	+++	+++	
III	2	-	-	-	+	+	-	-	4
	5	-	±	-	+++	+++	-	-	
IV	2	-	-	-	-	+	-	-	1
	5	-	-	-	-	+++	-	-	
V	2	-	-	+	+	+	-	-	4
	5	±	+++	+++	+++	+++	+++	+	
Control	2	+	+	+	+	+	+	+	
	5	+++	+++	+++	+++	+++	+++	+++	

Key: -, no growth; ±, very slight growth; +, slight but definite growth; +++, thick growth.

*Abbreviations not given in the footnote to Fig. 2: AICA, 5-amino-4-imidazole carboxamide; hyx, hypoxanthine; ade, adenine; xan, xanthine; gua, guanine.

in the purine biosynthetic pathway which form such a chromaphore have been described (Flaks and Lukens, 1963; Stouthamer et al., 1965; Mohapatra and Kloos, 1975). The culture supernatants from all of the other mutants were negative for the Bratton-Marshall test, indicating that none of the mutants accumulated AICAR, SAICAR or FAICAR. Note that CAIR spontaneously decomposes to AIR, so that mutants in step 8 (Fig. 2) would accumulate AIR (Stouthamer et al., 1965).

From the above results it was determined that the mutants were blocked as follows:

Group I: before the formation of AIR (i.e. before step 6; Fig. 2).

Group II: IMP dehydrogenase (step 14, Fig. 2).

Group III: between the formation of AIR and SAICAR (i.e. step 7 or step 8, Fig. 2). It is not readily apparent why these mutants were not stimulated by AICAR.

Group IV: adenylosuccinate synthetase (step 12, Fig. 2). This mutant could not have been deficient in adenylosuccinase (step 13), since this enzyme also catalyses the formation of AICAR from SAICAR (step 9). Therefore mutants deficient in adenylosuccinase would accumulate SAICAR (Stouthamer et al., 1965).

Group V: the site of the block in these mutants is unknown, since their leaky nature made analysis very difficult.

Since the mutants in Groups I and III grew on adenine but not on guanine, it is apparent that R. trifolii can convert adenine to guanine, but not vice versa.

3.2.4 Symbiotic properties of auxotrophic mutants

The symbiotic properties of the auxotrophic mutants are given in Table 7. Effective nodules were found on plants inoculated with certain purine-requiring strains, but in these cases bacteria recovered from the nodules had a purine-positive phenotype. Also, the inoculant strains were characterized by relatively high reversion frequencies ($\sim 10^{-6}$), unlike most of the purine-

Table 7 Symbiotic properties of auxotrophic mutants of R. trifolii

The symbiotic properties were evaluated 21-30 days after inoculation and are expressed relative to strain 7000 controls. At least two plants were used for each mutant tested. The plants were grown under a 16 h light period. In most cases, acetylene reduction tests were done to confirm visual observations. In all cases of effective nodulation, the phenotype of rhizobia from nodule crushes was tested.

Parent Strain	Requirement	Symbiotic property*				Total No.
		NN	NE	PE	E	
7000	glutamate-yielding amino-acid		1		1	2
7012	histidine				1	1
	leucine				2	2
	methionine				1	1
	nicotinic acid				3	3
	purine (Group I)	2		7	1***	10
	purine (Group II)	2		2		4
	purine (Group III)	1		2		3
	purine (Group V)			2	2***	4
7008	tryptophan	1				1
	uracil	3		1		4
7008** (trp-4)	choline				1	1
	methionine or cysteine				1	1
	purine (Group I)	1		1		2
	purine (Group IV)	1				1
	uracil or cytosine		1			1
7012 (his-6)	purine (Group I)	1		1		2
	purine (Group III)			1		1
	uracil			1		1

*NN, non-nodulating; NE, ineffective (< 5% acetylene reducing ability of strain 7000); PE, partially effective (5-20% acetylene-reducing ability of strain 7000); E, effective (similar amount of acetylene-reducing ability to strain 7000).

**Tryptophan-positive revertants of the mutant strains were used for nodulation tests.

***Bacteria isolated from these nodules had a purine-positive phenotype.

requiring strains isolated which were very stable (reversion frequency $<10^{-9}$). The nodules formed by the other nodulation-positive pur strains first appeared at least three days after the nodules formed by effective strains and gradually developed acetylene-reducing ability to a maximum of 20% of that of the wild-type strain. The development of the plants inoculated with the partially-effective pur strains was very poor and barely distinguishable from that of the uninoculated controls. The pur mutants which did not form nodules included representatives of groups I, III and IV, as defined in the preceding section; therefore the site of the block in the strains did not determine whether they were non-nodulating or partially-effective. The addition of adenine (5 $\mu g\ ml^{-1}$), or guanine (5 $\mu g\ ml^{-1}$) in the case of group II mutants to the growth medium of the plants allowed the non-nodulating pur mutants to form partially-effective nodules but had no effect on the nodulation properties of the other pur mutants.

The symbiotic properties of the pyrimidine-negative mutants were similar to those of the purine-negative mutants. Also, supplementation of the plant growth medium with uracil (5 $\mu g\ ml^{-1}$) permitted the non-nodulating pyr mutants to form partially-effective nodules, but had no effect on the properties of the partially-effective mutants.

The inability of the trp mutant 7008 to form nodules was interesting because it has been suggested that indoleacetic acid (IAA), which is derived from tryptophan, is involved in the initiation of nodule formation (reviewed by Libbenga and Bogers, 1974). A few nodules arose spasmodically on plants inoculated with strain 7008 and these nodules were deep pink and reduced considerable amounts of acetylene. Bacteria isolated from the nodules were tryptophan-negative, suggesting that nodulation by strain 7008 was impaired specifically at the stage of nodule initiation, i.e. once nodule initiation occurred, effective nodules

were formed. To determine whether this impairment was caused by an inability to synthesize IAA, the effect of the addition of IAA (final concentrations, 10^{-8} M, 10^{-6} M, 10^{-4} M) to the growth medium of plants inoculated with strain 7008 was determined. The effect of the addition of tryptophan (final concentrations, 10 $\mu g\ ml^{-1}$, 100 $\mu g\ ml^{-1}$) was also tested. The addition of 10^{-8} M IAA had no effect either on nodulation by strain 7008 or on plant growth. The other concentrations (10^{-6} M, 10^{-4} M) of IAA tested caused severe root deformations of the plants (stunting, 'lignification', no lateral roots) and no nodules were detected. The addition of either concentration of tryptophan had the same effect as observed for the higher concentrations of IAA.

The other auxotrophic mutants together with all str and rif mutants of strain 7000 tested formed effective nodules, with one exception, viz. one of the auxotrophic mutants requiring a glutamate-yielding amino-acid (strain 7056). However, the defective nodulation of this strain was not associated with its auxotrophic requirement (see Section 6.2.8). Thus overall the results showed that auxotrophic mutants requiring a nucleic acid base were defective in nodulation, whereas those requiring an amino-acid or vitamin were effective. The only exception to the latter class was strain 7008. Its inability to form nodules was associated with its auxotrophic requirement, since tryptophan-positive revertants of it nodulated red clover normally.

3.3 Discussion

The 'micro-colony' method provided a relatively efficient means of isolating auxotrophic mutants. The difference found in the growth of mutants on media solidified with Difco Bacto agar compared to media solidified with Difco Noble agar indicated that the rhizobia were very efficient scavengers of nutrients present as impurities in the former. This property may provide

an explanation for the failure to enrich for auxotrophs by ampicillin selection since lysis of the wild-type cells by ampicillin would release low concentrations of nutrient into the medium. The amount of these nutrients may have been sufficient to allow the auxotrophs originally present to grow and hence become susceptible to ampicillin.

The fact that the majority of mutants isolated by the micro-colony method required a nucleic acid base for growth may reflect an inherent property of the rhizobia or, more likely, the nature of the impurities present in the Difco Bacto agar. If the latter is the case, modification of the micro-colony method by adding to the medium a low amount of the compound for which the auxotrophs are required should facilitate their isolation. The feasibility of this approach was confirmed by plating a mixed culture of strains 7000 and 7008 on RDM-glucose (Difco Noble agar) plates containing 0.5 $\mu g\ ml^{-1}$ tryptophan. The *trp* mutants were readily distinguishable from the wild-type bacteria.

The analysis of the biochemical blocks in the purine-requiring mutants indicated that mutants in at least four different *pur* genes had been isolated. All purine or pyrimidine-requiring mutants obtained had defective symbiotic properties. Other workers have also found that purine- and pyrimidine-negative mutants of *Rhizobium* form a defective symbiosis (see Table 3; also review by Dénarié *et al.*, 1976). However, the significance of the invariable association between adenine requirement and ineffectiveness is open to question. Since adenine is a precursor in the biosynthesis of cytokinin which may be involved in the control of nodule development (Libbenga and Bogers, 1974; Syono *et al.*, 1976), it has been suggested that the ineffectiveness of adenine-requiring mutants results from their inability to synthesize cytokinin. However, the block in the symbiosis formed by many *pur* mutants occurs at a later stage than that at

which cytokinin is proposed to be involved (Dénarié et al., 1976). The fact that guanine- and pyrimidine- as well as adenine-negative mutants are ineffective suggests that the ineffectiveness has a more mundane causation. For examples, bacteroid production may demand a greater supply of purines and pyrimidines than can be satisfied by the host plant. The amount of free purine and pyrimidine in the host-cell cytoplasm may be very low, and/or purines and pyrimidines may not be excreted into the rhizosphere. It is also possible that the plasma-membrane which at all times separates the bacteria/bacteroids from the host cytoplasm is impermeable to the nucleic acid bases. This latter possibility is supported by the fact that an exogenous supply of adenine, etc. does not relieve the symbiotic block.

The finding that IAA did not allow nodulation of the tryptophan-negative mutant does not eliminate the possibility that the inability of this mutant to nodulate clover was caused by its inability to synthesize IAA. It has been proposed that IAA produced by the rhizobia is involved in softening the cell-wall of the root-hair prior to the penetration of the bacteria (see Section 1.3.3). If this is the case, the effect of IAA would be localized and therefore the addition of IAA to the whole root might not produce a similar effect.

3.4 Conclusions

A relatively efficient method of auxotrophic mutant isolation was developed and used to construct strains suitable for use in conjugation studies. The study of the symbiotic properties of the auxotrophic mutants generally confirmed the results of other workers.

CHAPTER 4

ATTEMPTS TO DEMONSTRATE CONJUGATION IN *R. trifolii*

4.1 Introduction

The development of a conjugation system in *R. trifolii* is of obvious importance for the genetic analysis of this species. At the time that this study was begun, chromosomal recombination had not been demonstrated in *Rhizobium*, although it had been shown that P-group R-factors were readily transmissable between strains of *R. leguminosarum* (Beringer, 1974). I had intended to use the R-factors RP4 and R68-44 (see Haas and Holloway, 1976) in attempts to demonstrate chromosomal recombination in *R. trifolii*. However, by the time suitable strains of *R. trifolii* were constructed, chromosomal recombination mediated by the P-group R-factor R68-45 had been reported in *R. leguminosarum* (Beringer and Hopwood, 1976). This plasmid, which was isolated from R68-44 on the basis of increased stability of its chromosome donor ability (Cda) (Haas and Holloway, 1976), was obtained from Beringer and used for the studies reported in this chapter. The strains used in this work are described in Table 8.

4.2 Results

4.2.1 Transfer of R68-45 from *E. coli* 1230 into strains W19 and 7000

The R-factor R68-45 specifies resistance to tetracycline, carbenicillin, kanamycin and neomycin. It was originally received in *E. coli* 1230, from which it was conjugated into *R. trifolii* strains W19 and 7000. Strain W19 was used since it was known not to contain any restriction-modification system (Salmond, personal communication). Kanamycin (50 μg ml^{-1}) was used to select for the transfer of R68-45 because the resistance to kanamycin specified by the R-factor is expressed well in *Rhizobium*. Also, the efficiency of plating (e.o.p.) of rhizobia containing R68-45 on media containing kanamycin is

higher than on media containing tetracycline or carbenicillin (Beringer, personal communications). The donor strain 1230 was counter-selected on the basis of its auxotrophy. The donor and recipient strains were also easily distinguished by their colony morphology.

Table 8 Strains used for the works described in Section 4.2

Strain*	Parentage	Characteristics
1230	-	*E. coli* K12 *pro met* (R68-45)
C603	-	*E. coli* C *arg his str*
7000 Kn^R(i)	1230 x 7000	7000 (R68-45)
7000 Kn^R(ii)	1230 x 7000	7000 (R68-45)
7000 Kn^R(iii)	1230 x 7000	7000 (R68-45)
W19 Kn^R(i)	1230 x W19	W19 (R68-45)
W19 Kn^R(ii)	1230 x W19	W19 (R68-45)
W1903	W19	*pur*-1 *rif*-1 *str*-1
7092	7000	*pur*-2 *rif*-2 *str*-2
7093	7000	*pur*-3 *rif*-3 *str*-3
7094	7000	*his*-6 *ura*-4
7095	7000	*trp*-4 *pur*-4 *str*-4
7097	7000	*trp*-4 *pur*-4 *rif*-4
6693	-	*R. meliloti rif*-293 (R68-45)

*All strains are *R. trifolii* unless otherwise stated

In the cross 1230 x W19, kanamycin-resistant recipients arose at a frequency of $\sim 1 \times 10^{-4}$ whereas in the cross 1230 x 7000, the frequency was $2\text{-}6 \times 10^{-5}$. Spontaneous kanamycin-resistant mutants of both recipient strains arose at a frequency of $\sim 1 \times 10^{-7}$. A number of kanamycin-resistant ex-conjugants (labelled Kn^R(i), Kn^R(ii), etc.) from each cross were purified by single-colony streaking and antibiograms were done for each (Table 9). That strain 7000 Kn^R(ii) had received R68-45 was also confirmed by crossing it with strain C603. The bacteria from the cross were diluted on to nutrient agar containing kanamycin (50 $\mu g\ ml^{-1}$) and

Table 9 Antibiograms of parental and recipient strains of R. trifolii and E. coli

The antibiograms were done as described in Section 2.5. Tetracycline and carbenicillin were contained on separate Oxoid antibiotic discs, and the other antibiotics were contained on a total of two Oxoid 'Multodiscs'. The results were read after incubation of the plates for two days, and are expressed as the radius (mm) of the cleared zone.

Antibiotic (μg on disc)	1230	7000	7000 Kn^R(i)	7000 Kn^R(ii)	7000 Kn^R(iii)	W19	W19 Kn^R(i)	W19 Kn^R(ii)	C603	C603 Kn^R(r)
kanamycin (30)	0	3	0	0	0	9	2	0	4	0
neomycin (30)	1	3	0	0	0	10	2	1	2	0
carbenicillin (100)	0	28	20	14	19	15	4	5	15	0
ampicillin (25)	0	22	11	7	11	9	2	2	12	0
tetracycline (50)	0	28	5	4	5	25	2	2	10	0
streptomycin (25)	6	7	6	6	7	16	17	18	0	0
rifampicin (30)	8	15	18	15	15	12	10	11	12	12
nalidixic acid (30)	0	14	14	14	14	0	0	0	14	12

Table 10 Efficiency of plating of strain 7000 Kn^R(ii) on various RDM-glucose plates containing various antibiotics

A culture of 7000 Kn^R(ii) grown in RDM-glucose broth was diluted for single colonies on RDM-glucose plates containing the specified antibiotics.

Antibiotic	Concentration ($\mu g\ ml^{-1}$)	Efficiency of plating (%)
none	-	100
kanamycin	100	100
tetracycline	5	40
tetracycline	10	2
carbenicillin	50	30-60

incubated at 37° C. Kanamycin-resistant recipients arose at a frequency of 2×10^{-3} and the one tested (C603 Kn^R(i)) expressed the appropriate resistance determinants (Table 9). The results showed that the resistances determined by R68-45 were expressed in R. trifolii, albeit relatively poorly in strain 7000. Strain 7000 Kn^R(ii) expressed the resistances somewhat better than the other strain 7000 derivatives, and its e.o.p. on RDM-glucose plates containing the various antibiotics was determined (Table 10, above). The results confirmed that kanamycin was the best antibiotic to use for selection of R. trifolii strains carrying R68-45.

4.2.2 Transfer of R68-45 between R. trifolii strains and attempts to demonstrate chromosomal recombination

In order to test for chromosomal recombination, advantage was taken of the exceptional stability of most pur auxotrophs. No adenine-positive revertants of W19 pur-1 were ever detected whereas strains 7000 pur-2 and pur-3 reverted to an adenine-positive phenotype at a frequency of $\sim 10^{-9}$. These purine-negative mutants were of independent origin as pur-2 was a Group I mutant and pur-3 a group III mutant. The three purine-negative mutants were doubly marked with rifampicin-resistance (rif) and

streptomycin-resistance (str) to provide recipient strains. Strain W1903 was used as recipient for crosses with strains W19 Km^R(i) and W19 Km^R(ii) and strains 7092 and 7093 as recipients in crosses with strains 7000 Km^R(i), 7000 Km^R(ii) and 7000 Km^R(iii). The large number of donor strains were used in case the R-factor in some of them had lost its Cda property during transfer from 1230. The selective medium used was RDM-glucose containing rifampicin and streptomycin only, i.e. no selection was made for R68-45 transfer, but the donor strain was counter-selected by both rifampicin and streptomycin. However, in all crosses, purine-positive colonies arose only at the frequency characteristic of the reversion of the pur allele being selected, i.e. with W1903 as recipient, no such colonies were found, whereas in crosses with 7092 and 7093 the frequency of purine-positive colonies was $\sim 10^{-9}$. In all crosses, kanamycin-resistance was transferred at a frequency of $\sim 10^{-2}$. Of the eight purine-positive colonies found in crosses involving strains 7092 or 7093, only two had gained the R68-45 resistance determinants. Therefore no evidence for R68-45 mediated recombination was obtained in these experiments.

Johnston and Beringer (1977) reported that R68-45 promoted chromosomal recombination between R. leguminosarum and R. trifolii in either direction, and that rif and str were closely linked in both species. Hence co-transfer of rif and str would provide a good test for genetic recombination. Another isolate of R68-45, contained in R. meliloti strain 6693 (Johnston et al., 1978b), was obtained and transferred to strain 7092 (frequency $\sim 10^{-2}$). In this case, 5 independent crosses were made, each using a separate single colony of 6693 to produce the donor culture, and 4 single colonies of 7092 containing R68-45 were selected from each cross. Each of these twenty strains was then used as a donor in a cross with strain 7094. The selective medium used was RDM-glucose containing histidine, uracil, rifampicin and streptomycin. The colonies which appeared were then tested for the presence of the

his-6 and ura-4 alleles. Invariably these colonies were found to be His^+ and Ura^+ and hence were probably purine-positive revertants of strain 7092, i.e. not even a single recombinant from more than 2×10^{10} bacteria plated was found. Note that these experiments depended upon the assumption that rif and str were closely linked in R. trifolii 7000.

4.2.3 Attempts to isolate R-primes in R. trifolii 7000

Johnston et al. (1978b) reported that recombinants obtained using R. meliloti as donor and R. leguminosarum as recipient donated the selected allele at high frequency. They found that these recombinants harboured R-prime factors carrying the selected allele from R. meliloti. In my study, analogous experiments were done using R. meliloti 6693 as donor (Johnston et al., 1978b) and str derivatives of the doubly auxotrophic mutants of R. trifolii 7000 and also W19 pur-1 as recipients. In these experiments only one of the auxotrophic markers was counter-selected and no selection was made for the transfer of R68-45. The donor strain was counter-selected by incorporation of streptomycin in the medium. The vast majority of colonies obtained on the selective plates were streptomycin-resistant derivatives of strain 6693 (frequency $\sim 10^{-7}$) as judged by their ability to grow on nutrient agar. Unfortunately it was difficult to differentiate between the colonial morphology of strain 6693 and R. trifolii and so no rapid method for identifying R. trifolii recombinants was available. However, one purine-positive derivative of strain 7095 was obtained and labelled strain 7096. This strain grew very slowly in the absence of adenine and contained R68-45, suggesting that it may have obtained its pur^+ allele from R. meliloti. However, when it was crossed with strain 7097, no increase in the number of purine-positive colonies was obtained although the kanamycin-resistance was transferred at a frequency of $\sim 10^{-2}$. Upon sub-culturing of strain 7096, the strain reverted to a fast-growing phenotype, characteristic of a true revertant.

4.3 Discussion

The inability to demonstrate chromosomal recombination in *R. trifolii* 7000 was very disappointing. It may reflect an inherent property of the strain, and indeed other workers have found that not all strains of *R. trifolii* are able to act as recipients for the *rif* and *str* genes from *R. leguminosarum* (Johnston, personal communication). A second alternative is that the isolates of R68-45 used lost their Cda property upon transfer to *R. trifolii* 7000. The Cda property is unstable in *Rhizobium* as well as in other genera, and it has been found that up to 50% of recombinants formed by R68-45 mediated recombination in *R. leguminosarum* (Johnston, personal communication) and *R. meliloti* (Kondorosi *et al.*, 1977) are unable to act as chromosome donors.

The remarkable progress in the study of *Rhizobium* genetics which has been made over the past three years was reviewed in Section 1.9. It was partly this progress, together with the lack of success gained, that led to more effort being devoted to the studies described in the other chapters of this thesis rather than to furthering those described in this chapter. However, the work in this chapter has led to strains being available to exploit any future progress that may be made in this field.

4.3 Discussion

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CHAPTER 5

STUDIES ON NITROGEN ASSIMILATION BY RHIZOBIA

5.1 Introduction

Knowledge, prior to this work, on nitrogen assimilation and its control in free-living and bacteroid forms of rhizobia has been discussed (Section 1.7.2). It was thought that the mechanism of nitrogen assimilation in free-living rhizobia was similar to that of enteric bacteria, with the GS/GOGAT pathway functioning at low ammonia* concentrations and the GDH pathway at high ammonia concentrations. Rhizobial enzymes were thought to be insignificant in the assimilation of ammonia in the nodule, but their role, if any, in the regulation of nitrogenase was unknown.

The aim of this work was to isolate mutants of R. trifolii deficient in GS or GOGAT. They were to be used to investigate the role of these enzymes in nodule function, and also in the regulation of nitrogen assimilation in free-living rhizobia.

5.2 Results

5.2.1 Studies on the growth of strain W19

The growth response of strain W19 to the amino-acid pools (see Section 3.2.1) suggested that it had a defect in ammonia assimilation. Growth of W19 on RDM-glucose containing one of aspartate, glutamate, histidine or proline was obtained in broths or in lawns, but only after 5 to 6 days incubation, whereas control cultures in YM medium grew by 2 days. Growth was not obtained in RDM-glucose containing any other amino-acid, including glutamine and arginine, nor if the medium was supplemented with succinate. The growth of strain W19 when diluted for single colonies on a variety of solid media is shown in Table 11. Growth on RDM-mannitol solidified with Difco Noble agar and supplemented with proline or glutamate was initially very poor but 'large' derivatives arise spasmodically from the small colonies and were frequent by 10 days. These large derivatives

* In this thesis, the term 'ammonia' is used to denote both NH_3 and NH_4^+

Table 11

Growth of strain W19 on various solid media

All RDM media contained mannitol and 3.5 mM NH_4Cl. Proline and glutamate were added where specified to 100 $\mu g\ ml^{-1}$. Each plate contained approximately 100 colonies.

Medium*	Colony diameter			
	Days after inoculation			
	3	4	6	10
YMA	0.8 mm	3.0 mm	-	-
RDM-N	specks	0.1 mm	0.4 mm	1.0 mm
RDM-B	0.1 mm	0.4 mm	1.2 mm	1.8 mm
RDM-N + proline	specks	0.1 mm	most 0.4 mm, few 1.0 mm	most 1.2 mm, few 3.0-5.0 mm
RDM-B + proline	0.2 mm	0.6 mm	1.0 mm	1.8 mm
RDM-N + glutamate	specks	0.4 mm	most 1.0 mm, few 2.0 mm	most 1.8 mm, ~5% 5.0 mm
RDM-B + glutamate	0.1 mm	0.7 mm	1.2 mm	2.0 mm

* Abbreviations: YMA, yeast mannitol agar

RDM-N, defined medium solidified with Difco Noble agar

RDM-B, defined medium solidified with Difco Bacto agar

were not seen if the medium was solidified with Difco Bacto agar. One large (W19 pro L) colony and one small (W19 pro S) colony were picked from the RDM-N-mannitol plates containing proline and purified by 3 cycles of streaking for single colonies on GSYC. A single colony from each isolate was then grown in YM broth and diluted for single colonies on various defined media (Table 12). The aim of the experiment was to compare the growth of the derivatives on glutamate, histidine and proline and to test the effect of ammonia concentration on growth. The effect of ammonia was tested because ammonia normally represses the synthesis of enzymes responsible for the utilization of glutamate, histidine and proline in K. aerogenes (Magasanik et al., 1974). The difference in the results shown in Tables 11 and 12 may have been due to the age of the inocula used. The experiments in Table 11 were done with a YM broth culture that had been in the stationary phase of growth for 24 h before use, whereas the inocula used for experiments in Table 12 were in late-exponential phase.

Four observations were made from the data in Table 12 : (i) no difference was observed between plates containing histidine or proline; (ii) W19 pro L, isolated on proline, grew better than W19 pro S on media containing proline, glutamate or histidine; (iii) the presence of 3 mM NH_4Cl in the medium had little effect on growth; (iv) the presence of 20 mM NH_4Cl in the medium suppressed the growth of both derivatives on all three amino-acids. The effect of the high ammonia concentration was also apparent on plates containing no amino-acids. After 8 days, colonies on the 'no nitrogen' and 'low NH_4^+' plates were 1.0 mm in size, whereas those on the 'high NH_4^+' plates were 0.2 mm.

Mutants of W19 pro S, able to grow with NH_4Cl as sole N source, were isolated by spreading 10^8 bacteria on to the surface of unsupplemented RDM-mannitol plates containing 3 mM NH_4Cl. Two colonies were found only after extended incubation (30 days) of the five plates. No colonies were found on two similar plates to which

Table 12

Growth of W19 pro S and W19 pro L on RDM-mannitol containing various nitrogen sources

Cultures were grown in YM broth, washed once in distilled water and diluted and spread to give approximately 50 colony forming units per plate. The medium used was RDM (Difco Noble agar) containing mannitol and the specified nitrogen source.

Nitrogen source*	Colony diameter after 4 days	
	W19 pro S	W19 pro L
no nitrogen low NH_4^+ high NH_4^+	colonies visible as specks (0.1 mm)	as for W19 pro S
proline or histidine	0.1 mm	2.0 mm
low NH_4^+ + proline or histidine	0.1 mm	1.5 mm
high NH_4^+ + proline or histidine	0.1 mm	0.8 mm
glutamate	variable colony size, up to 0.8 mm	2.0 mm
low NH_4^+ + glutamate	typical colony size 1.2 mm	2.0 mm
high NH_4^+ + glutamate	0.3 mm	0.8 mm

* low NH_4^+, 3 mM NH_4Cl; high NH_4^+, 20 mM NH_4Cl; proline, 100 $\mu g\ ml^{-1}$; glutamate, 100 $\mu g\ ml^{-1}$; histidine, 100 $\mu g\ ml^{-1}$

a crystal of NTG had been added. The two 'revertants' were labelled W19 aut + 1 and W19 aut + 2 (aut = ammonia utilization). Growth of W19 aut + 1 on various solid media is compared to that of W19 pro L in Table 13 . W19 aut + 2 showed similar growth to W19 aut + 1. Strain W19 aut + 1 was able to grow slowly on RDM-glucose with 3 mM NH_4^+Cl as sole N source, but was inhibited by 20 mM NH_4Cl. The ability of W19 aut + 1 to use amino acids as sole N source was compared to that of W19 pro L and also other R. trifolii strains (Table 14). The results indicated that R. trifolii strains in general were able to utilize most of the amino acids as sole N source, strains W 19 pro L and 7049-L being the only exceptions. Glycine was toxic to all R. trifolii strains. The mutants of W19 able to grow on ammonia gained the ability to utilize most amino acids as sole N source, with alanine, arginine, asparagine, glutamine, glycine, methionine and serine the only exceptions.

The inability of strain W19 to grow with low or high concentrations of ammonia as sole N source suggested that it lacked both GOGAT and GDH. The inability of W19 pro S to grow with various amino-acids, including those directly convertible to glutamate, as sole N source, was similar to that found in GOGAT-negative mutants of Klebsiella (Nagatani et al., 1971; Brenchley et al., 1973) and E. coli (Berberich, 1972). Revertants of GOGAT-negative mutants of K. aerogenes that are able to grow on the amino-acids convertible to glutamate produce GS constitutively (Brenchley et al., 1973; Prival et al., 1973). Therefore W19 pro L could be a GS constitutive mutant of W19 pro S. The repressive effect of high concentrations of ammonia on the growth of W19 pro S and W19 pro L suggested that GS activity was repressible by ammonia in both strains. Therefore the enzymatic lesions in the strain W19 derivatives were determined. Strain 7000, which was able to grow with low or high ammonia or most amino-acids as sole N source, was used as a control.

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Table 13

Growth of W19 aut + 1 and W19 pro L on various solid media

All media were solidified with Difco Noble agar. Cultures were grown in YM broth and diluted to give approximately 70 colonies per plate. The data is from one experiment, but two other experiments gave similar results. The values represent average colony diameter in mm.

Medium	Days after plating					
	4		5		7	
	pro L	aut + 1	pro L	aut + 1	pro L	aut + 1
YM	2.2	2.0	-*	-	-	-
GSYC	2.2	2.0	-	-	-	-
RDM-glucose + 3 mM NH_4^+	0.2	0.2	0.4	0.6	1.0	2.5
RDM-glucose + 3 mM NH_4^+ + glutamate	1.2	0.6	3.0	2.2	-	-
RDM-glucose + 20 mM NH_4^+	0.2	0.2	0.4	0.5	0.8	1.2

* not noted

Table 14 Ability of R. trifolii strains to use various amino-acids as sole N source

Nitrogen source	Strain									
	W19 pro L	W19 aut + 1	W19 aut + 2	IDL	P2	SU297	SU298	CR 5	7000	7049-L
NH_4Cl (3 mM)	-	+	+	+	+	+	+	+	+	-
alanine	-	-	-	+	+	+	+	-	+	-
arginine	-	-	-	+	+	+	+	-	+	+
asparagine	-	-	-	+	+	+	±	-	+	+
aspartate	+	+	+	+	+	+	+	+	+	+
cysteine	-	+	+	-	+	+	+	+	+	-
cystine	-	+	+	+	+	+	+	+	+	-
glutamate	+	+	+	+	+	+	+	+	+	+
glutamine	-	-	-	+	+	+	+	+	+	+
glycine	-	-	-	-	-	-	-	-	-	-
histidine	+	+	+	-	+	+	+	+	+	+
hydroxyproline	+	+	+	+	+	+	+	+	+	+
isoleucine	-	+	+	+	+	+	+	+	+	-
leucine	-	+	+	+	+	+	+	+	+	-
lysine	-	±	+	+	+	+	+	+	+	-
methionine	-	-	-	+	+	+	+	+	+	-
phenylalanine	-	±	±	+	-	+	-	+	+	-
proline	+	+	+	+	+	+	+	+	+	+

(table continued)

Table 14 (continued)

Nitrogen source	Strain									
	W19 pro L	W19 aut + 1	W19 aut + 2	IDL	P2	SU927	SU928	CR5	7000	7049-L
serine	-	-	-	+	+	+	-	-	+	-
threonine	-	+	+	+	+	+	+	+	+	-
tryptophan	-	+	±	-	-	+	-	-	+	-
tyrosine	-	+	+	+	+	+	+	+	+	-
valine	-	+	+	±	±	+	+	±	+	-

Single colonies of the strains were picked from fresh YM plates with a toothpick, and patch-streaked on to RDM-glucose plates containing the specified amino-acid (50 μg ml^{-1}) as sole N source. The experiment was repeated 3 times with the plates streaked in different order to avoid 'inoculum effects'. One 'no nitrogen' plate, four 'amino-acid' plates and one GSYC plate were streaked sequentially with each toothpick. The amount of growth was recorded after 4 days incubation.

\+ Thick growth compared to 'no nitrogen' control

± Slight growth compared to 'no nitrogen' control

\- No growth compared to 'no nitrogen' control

5.2.2 The presence of GOGAT and GDH in R. trifolii strains

Crude extracts prepared from strains W19 pro L, W19 pro S, W19 aut + 1 and 7000 grown in a variety of media were examined for GDH, GOGAT and alanine dehydrogenase activities. Both NADH- and NADPH-dependent activities were assayed. In no case was GDH or alanine dehydrogenase activity found. However, NADH oxidase activity was present in the extracts and may have masked low amounts of NADH-dependent activity. No GOGAT activity was found in strains W19 pro S or W19 pro L, but GOGAT activity specific for NADPH was found in strain 7000. The amount of this activity was relatively constant irrespective of the medium used for growth. A very low amount of GOGAT activity but no GDH activity was found in W19 aut + 1 (Table 15).

Brown and Dilworth (1975) reported that R. trifolii strain WU1 contained biosynthetic GDH"when grown in the presence of an ammonia excess". Therefore, my inability to find GDH when strain 7000 was grown with 20 mM NH_4Cl as sole N source was surprising. I therefore tested other R. trifolii strains for GDH. Extracts were prepared from various R. trifolii strains, one R. leguminosarum strain and 2 Klebsiella strains grown in RDM containing 0.1% (w/v) glucose and 20 mM NH_4Cl. All Rhizobium strains contained GOGAT activity but none, including WU1 used by Brown and Dilworth, contained GDH activity. Both Klebsiella strains contained GDH and one contained GOGAT (Table 16). Addition of R. trifolii extract to Klebsiella extracts had no effect on the specific activity of the GDH from Klebsiella.

Brown and Dilworth (1975) found GDH in strain WU1 when it was grown in chemostat culture under glucose limitation in the presence of excess ammonia. We attempted to grow strain 7000 under similar conditions, but were defeated by equipment breakdown and contamination problems. Nevertheless, unlike us, Brown and Dilworth (1975) did find GDH in batch cultures of a R. leguminosarum strain.

Table 15

GOGAT activity in extracts prepared from R. trifolii strains W19 pro L, W19 pro S, W19 aut + 1 and 7000 cultured on various media

Medium	NADPH-dependent GOGAT activity ($nmol^{-1}(mg\ protein)^{-1}$)			
	W19 pro L	W19 pro S	W19 aut+1	7000
YM	0	0	-*	30
GSYC	0	0	5	39
RDM-glucose, 1 mM NH_4^+	-*	-	5	18
RDM-glucose, 20 mM NH_4^+	-	-	-	23
RDM-glucose, 1 mM NH_4^+, 10 mM histidine	0	-	5	36
RDM-glucose, 20 mM NH_4^+, 10 mM histidine	0	-	-	37
RDM-glucose, 0.1% (w/v) casein hydrolyzate	0	0	7	30

* not done

Table 16

GDH and GOGAT activities in R. trifolii, R. leguminosarum and Klebsiella strains

Cultures were grown in RDM containing 0.1% (w/v) glucose and 20 mM NH_4Cl, and were assayed for NADP-dependent GDH and GOGAT as described in Chapter 2.

Strain	Specific activity (nmol min^{-1} (mg protein)$^{-1}$)	
	GOGAT	GDH
R. trifolii		
7000	36	0
CR5	19	0
FA6	50	0
IDL	36	0
P2	40	0
VW1	10	0
WU1	23	0
R. leguminosarum		
1036	42	0
Klebsiella		
CI	70	180
MK53	2	375

Thus enzyme assays indicated that all R. trifolii strains lacked GDH. Strain W19 also lacked GOGAT, but the other R. trifolii strains possessed GOGAT even under high ammonia conditions. Strain W19 aut + 1 regained GOGAT, albeit at very low levels, which may explain the slow growth of W19 aut + 1 on low ammonia. Attempts to isolate faster-growing derivatives of W19 aut + 1 by serial culture in RDM-glucose + 3 mM ammonia were unsuccessful.

Strains W19 pro S, W19 pro L and W19 aut + 1 all formed ineffective nodules on the 2 cultivars (Attaswede and Kenland) of red clover and one cultivar (Mt. Barker) of subterreanean clover tested. Therefore no conclusions on the role of rhizobial GOGAT in nodule function could be made. Forty strains of R. trifolii, isolated by V. Waddell from clover nodules obtained from various sites in Warwickshire, were examined for growth on RDM-glucose containing 20 mM or 1 mM ammonia as sole N source. All were able to grow on both media and therefore to assimilate ammonia. It was therefore decided to isolate GOGAT negative mutants of strain 7000 which was effective on red clover.

5.2.3 Attempts to isolate GOGAT and GS mutants of R. trifolii 7000

The medium used for mutagenesis and non-selective growth of strain 7000 was RDM-glucose supplemented with 1 mM NH_4Cl, 100 $\mu g\ ml^{-1}$ glutamine and 100 $\mu g\ ml^{-1}$ glutamate. The medium used for ampicillin enrichment was RDM-glucose containing 1 mM NH_4Cl as sole N source. These media were selected in the hope of isolating both GOGAT and GS negative mutants. The mutagenesis and enrichments were done as described in Materials and Methods (Section 2.4). Six independent experiments, each including 3 ampicillin enrichments, were done. Bacteria were plated out after each enrichment. Approximately thirty mutants were found, but all were able to grow if the medium was supplemented with succinate.

A subsequent mutagenesis experiment with succinate included in the enrichment medium failed to give any glutamate- or glutamine-dependent mutants, although further succinate-dependent mutants were found. At about this time, other workers described GS and GOGAT

negative mutants of rhizobia (Kondorosi et al., 1977; Ludwig and Signer, 1977) and no further attempt was made to isolate GS or GOGAT negative mutants of R. trifolii 7000.

5.2.4 Growth and symbiotic properties of succinate-dependent mutants of R. trifolii 7000

Two strains were used for most of this work. Strain 7049 was one of the succinate-dependent mutants isolated as described in the previous section, but strain 7056 was obtained by a procedure used to isolate non-specific auxotrophs (see Section 3.2.2). The growth of these two succinate-dependent mutant strains on plates containing various amino-acids in the place of succinate was tested. Their growth on RDM-glucose plates containing glutamate, histidine or proline, plus or minus low ammonia, resembled that of strain W19 pro S. Growth was initially very poor on these amino-acids, but large derivatives arose at high frequency (50% by 12 days) from the small colonies. Growth on RDM-glucose containing succinate or glutamine was at the same rate as strain 7000, suggesting that the synthesis of glutamine limited growth of the mutant strains on amino-acids normally converted to glutamate. This suggested that GS was repressed when the strains were grown on the other amino-acids. A large derivative of each strain was able to grow on RDM-glucose supplemented with one of arginine, aspartate, asparagine, glutamate, glutamine, histidine, hydroxyproline or proline only (e.g. strain 7049-L; Table 14). The addition of 20 mM NH_4^+ to media containing histidine, proline or glutamate had no effect on the growth of strain 7049-L, in contrast to the effect observed with W19 pro L. A revertant of strain 7049-L was isolated on RDM-glucose containing 2 mM NH_4Cl as sole N source, labelled 7084, and examined for GS activity (see Section 5.2.5). Strains 7049 and 7056 contained GOGAT activity but lacked pyruvate carboxylase. They are further described in Section 6.2.7.

The symbiotic properties of the various strains described above were then tested. Strains 7000, 7049, 7049-L and the other succinate-dependent mutants, except strain 7056, formed fully effective nodules. Strain 7056 formed ineffective nodules, but pyruvate-carboxylase-positive revertants of it were still ineffective (see Section 6.2.8). Nodulation by rhizobia is inhibited by fixed nitrogen. The addition of 0.5 mM histidine to the N-free seedling solution at the time of inoculation inhibited nodulation by strains 7000, 7049, 7049-L and 7056, indicating that the mutation which allowed strain 7049-L to grow on the various amino-acids did not allow nodulation in the presence of histidine. It had been hoped that if 7049-L was a GS-constitutive strain, histidine may not have inhibited its nodulation. Unfortunately the effect of the addition of a fixed nitrogen source on acetylene reduction by effective nodules of strain 7049-L was not tested at the time these experiments were done. Such an experiment might have been worthwhile because fixed nitrogen, as well as inhibiting nodulation, causes loss of acetylene reduction ability when added to the rooting medium of effectively-nodulated plants (e.g. various papers in Hardy and Gibson, 1977).

5.2.5 Regulation of GS and histidase in *R. trifolii*

The lack of GDH and presence of GOGAT under high ammonia conditions suggested, in contrast to the results of Brown and Dilworth (1975) that *R. trifolii* regulated its ammonia assimilatory enzymes in different fashion to that described for enteric bacteria. Therefore the activity of GS in various *R. trifolii* strains under a variety of growth conditions was examined. In *K. aerogenes*, GS regulates the formation of histidase, the first enzyme in the histidine catabolic pathway, in response to ammonia concentration (Magasanik *et al.*, 1974). Therefore the effect of ammonia on the amount of histidase synthesized was also investigated as a model system for an enzyme regulated by GS in *K. aerogenes*.

Glutamine synthetase was assayed using the transferase assay under conditions developed for the enzyme from E. coli. The average state of adenylylation of GS was calculated, using the formula: $\bar{n} = 12\text{-}12\,(\frac{b}{a})$

where $\bar{n}$ = average number of subunits of GS adenylylate;

a = the amount of γ-glutamyltransferase activity in the presence of 0.3 mM $MnCl_2$;

b = the amount of γ-glutamyltransferase activity in the presence of 0.3 mM $MnCl_2$ plus 60 mM $MgCl_2$.

However, it should be noted that no evidence for adenylylation of rhizobial GS was looked for, and the difference found in activity in the presence versus the absence of Mg^{2+} may reflect some factor other than adenylylation of the GS. The transferase assay measures GS activity in the reverse direction to that in which the enzyme functions under physiological conditions and so a biosynthetic assay in the presence of Mg^{2+} (Elliot, 1955), measuring GS in the forward direction, was also done in later experiments.

The GS activities found in various R. trifolii strains grown in RDM-glucose containing various nitrogen sources are shown in Table 17. Histidase activities are included where these were determined. Because of the large variability found between experiments, each line contains the results from a single experiment. However, no clear-cut correlation of GS transferase activity or $\bar{n}$ value with ammonia concentrations was found with any of the strains used. Also, GS activities obtained using the biosynthetic assay correlated poorly with those obtained using the transferase assay. It was therefore difficult to draw any conclusions regarding GS activity from the data. Especially disappointing was the failure to find any clear-cut difference between the GS activities of W19 pro L or 7084 and the other strains. Taken overall, the GS transferase activity data suggested that rhizobial GS was inhibited approximately 50% by the presence of Mg^{2+}, regardless of the nitrogen source in the medium used for growth of the bacteria. However, all R. trifolii

Table 17 Glutamine synthetase and histidase activities found in crude extracts prepared from R. trifolii strains grown in RDM-glucose containing various nitrogen sources

Extracts were prepared and assayed as described in Sections 2.6 and 2.9.2.4. Each line contains the results from a single experiment. Each value is the mean of duplicate determinations.

Strain	N source(s) in growth medium	Glutamine synthetase activity: Biosynthetic assay	Transferase assay: Mn^{2+}	Transferase assay: $Mn^{2+}+Mg^{2+}$	$\bar{n}$	Histidase activity
W19 pro L	1 mM NH_4^+ + 0.5 glutamate		550	360	4.0	
			620	300	6.2	
	0.5 mM glutamate		540	315	5.0	
	20 mM NH_4^+ + 0.5 mM glutamate		320	390	-2.8	
			450	120	8.9	
	10 mM histidine		630	230	7.6	72
P2	1 mM NH_4^+ + 0.5 glutamate		760	110	9.3	
	20 mM NH_4^+ + 0.5 mM glutamate		1290	110	11.0	
IDL	1 mM NH_4^+		770	220	8.6	
			850	730	1.7	
	20 mM NH_4^+		890	110	10.5	
			450	380	2.5	
			690	190	8.7	
WU1	1 mM NH_4^+		500	180	7.7	0.8
	20 mM NH_4^+		490	90	9.8	
	10 mM histidine	99	290	140	6.1	55
	1 mM NH_4^+ + 10 mM histidine	57	270	70	8.9	60
	20 mM NH_4^+ + 10 mM histidine	176	310	90	8.5	32
7000	1 mM NH_4^+	105	750	1100	-5.6	1.5
		80	450	200	6.7	
			750	410	5.4	
	20 mM NH_4^+	50	550	230	7.1	
		35	500	110	9.3	1.0
	10 mM histidine	65	410	250	4.6	42
		17	580	75	10.4	86
	1 mM NH_4^+ + 10 mM histidine	80	280	75	8.7	36
	20 mM NH_4^+ + 10 mM histidine	30	390	70	9.8	44
7084	1 mM NH_4^+		760		6.8	
			620		6.2	
	20 mM NH_4^+		640		6.4	
	10 mM histidine		410		4.6	45

strains tested, using either assay, contained readily measurable amounts of GS activity, even when cultured under conditions of ammonia excess. This was in contrast to the results of Brown and Dilworth (1975) who found that GS was completely absent when the rhizobia were cultured in a chemostat under glucose limitation and nitrogen excess. Our attempts to grow R. trifolii 7000 under similar conditions were unsuccessful (see Section 5.2.2).

For comparative purposes, similar experiments were done using batch cultures of various Klebsiella strains (Table 18). In all three strains, the activity obtained using the transferase assay showed that the GS was repressed and adenylylated under high ammonia conditions. The activity obtained with the biosynthetic assay was also much less when the bacteria were cultured under high ammonia conditions than when they were cultured under low ammonia conditions. Thus these results were similar to those previously reported for Klebsiella (e.g. Brenchley et al., 1973). The histidase activities of strains CI and MK53 were also repressed under high ammonia conditions as reported by Neidhardt and Magasanik (1957), provided that the bacteria were harvested when in the exponential phase of growth (Table 18). Cultures harvested when in the stationary phase of growth contained derepressed amounts of histidase irrespective of the presence of ammonia (data not shown). However, when strain A was cultured in the presence of histidine, its GS was highly adenylylated and the amount of histidase was unaffected by either the presence of ammonia in the medium or the growth phase of the bacteria. Thus the control of the histidine utilization genes in this strain was exceptional. However, overall the results shown in Table 18 confirmed published work, suggesting that the failure to find similar fluctuations in the R. trifolii strains may not have been due to the procedures used.

The histidase data in Table 17 suggested that in R. trifolii strains 7000 and WU1, histidase, while induced by the presence of

Table 18 Glutamine synthetase and histidase activities in extracts prepared from various Klebsiella strains grown in RDM-glucose containing various nitrogen sources

Bacteria were grown in 500 ml broths contained in 2 l Erlenmeyer flasks. Broths were inoculated with 0.5 ml of an overnight culture and grown for 10 h before harvesting.

Strain	N source in growth medium	Glutamine synthetase activity			$\bar{n}$	Histidase activity
		Biosynthetic assay	Transferase assay			
			Mn^{2+}	$Mn^{2+}+Mg^{2+}$		
CI-ONE	1mM NH_4^+		510	470	0.9	
	20mM NH_4^+		310	0	12.0	
	1mM NH_4^+ + 10mM histidine		540	400	3.2	34
	20mM NH_4^+ + 10mM histidine		260	0	12.0	6
A	1mM NH_4^+	1563	540	390	3.3	2
	20mM NH_4^+	550	310	0	12.0	0.5
	10mM histidine	120	760	40	11.3	108
	1mM NH_4^+ + 10mM histidine	280	2220	170	11.1	103
	20 mM NH_4^+ + 10mM histidine	30	270	0	12.0	117
MK 53	10 mM histidine	3045	800	830	-0.45	75
	1mM histidine	2700	880	730	2.0	60
	20mM NH_4^+	250	240	0	12.0	6

histidine in the medium, was not repressed by ammonia. The data in Table 18 suggested that the sensitivity of histidase to ammonia repression may be a strain-specific property. Therefore, a number of R. trifolii strains were examined for histidase activity after growth in RDM-glucose containing 20 mM NH_4^+ plus 10 mM histidine. All cultures were harvested when in the exponential phase of growth. Each strain examined contained high amounts of histidase activity under these conditions (Table 19).

Table 19

Histidase activities in R. trifolii strains

	7000	WU1	FA6	IDL	P2	W19proL	VW1	CR5
Histidase (nmol min^{-1} (mg protein $)^{-1}$)	44	32	102	85	40	60	96	210

In enteric bacteria, glucose exerts strong catabolite repression on histidase but succinate does not (Magasanik et al., 1965). However, in P. aeruginosa succinate exerts strong catabolite repression on histidase but the repression exerted by glucose is relatively weak (Lessie and Neidhardt, 1967). In both cases the strong catabolite repression is relieved by ammonia limitation (Neidhardt and Magasanik, 1957; Prival and Magasanik, 1971; Potts and Clarke, 1976). Therefore the possibility remained that histidase in R. trifolii was released from catabolite repression in the RDM-glucose medium used, although none of the R. trifolii strains listed in Table 19 grew with histidine as sole carbon source, in the presence or absence of ammonia (Primrose, personal communication). The effect of succinate on histidase activity in strain 7000 was then examined. Incubation of strain 7000 in media containing succinate as sole carbon source apart from histidine or in any media containing more than 5 mM succinate resulted in clumping and lyzing of the bacteria. However, the bacteria grew in media containing glucose plus 5 mM succinate, but the presence of succinate had no effect upon the amount of histidase activity found.

That histidase in strain 7000 was not repressed by ammonia was also indicated by the growth curve observed with a histidine auxotroph, derived from strain 7000, growing in RDM-glucose broth containing 20 mM NH_4Cl, 0.5 mM histidine and 0.5 mM tryptophan. The growth curves observed with strains 7012 (his) and 7008 (trp) in this medium are shown in Fig. 3. The growth of strain 7012 ceased at approximately 50% of the cell density obtained with strain 7008, but recommenced if further histidine was added. Since the amount of histidine originally in the medium was in vast excess of that normally needed to satisfy an auxotrophic requirement, this experiment suggested that the histidine was degraded during growth. Since strain 7000 can also utilize tryptophan as sole nitrogen source (Table 14), the experiment also suggested that the genes required for tryptophan catabolism were not expressed under the conditions used.

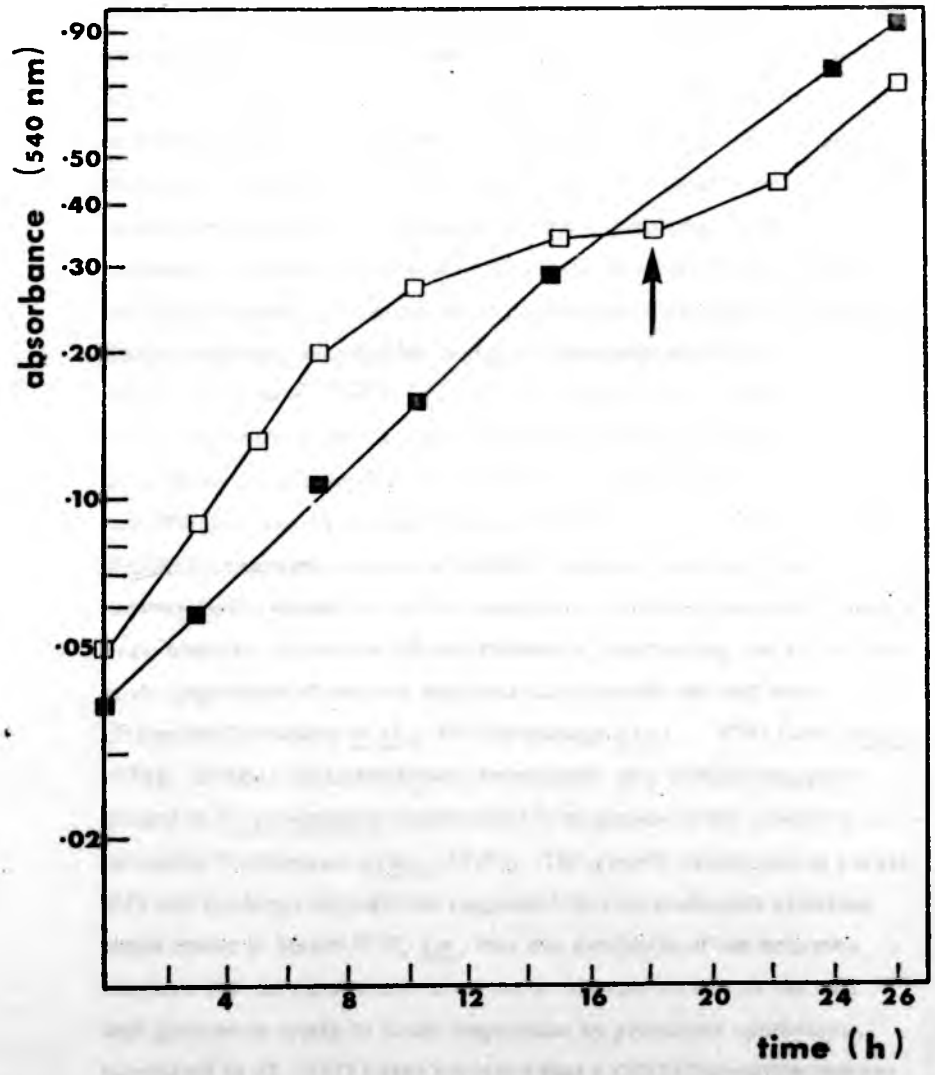
5.3 Discussion

No evidence for the presence of biosynthetically-active GDH or alanine dehydrogenase in R. trifolii was found, suggesting that the species assimilated ammonia solely by the GS/GOGAT pathway. The inability of R. trifolii strain W19, which was found to lack GOGAT as well as GDH, to utilize ammonia as nitrogen source provided further evidence that the GS/GOGAT pathway was the sole route of ammonia assimilation in the species. GOGAT-negative mutants of enteric bacteria are able to utilize high concentrations of ammonia because they possess GDH (Nagatani et al., 1971; Berberich, 1972; Brenchley et al., 1973; Pahel et al., 1978). However, a GOGAT negative mutant of R. meliloti was, like W19, unable to utilize ammonia as sole N source (Kondorosi et al., 1977). The latter workers and others were also unable to find biosynthetic GDH in Rhizobium (O'Gara and Shanmugam, 1976, 1977; Kondorosi et al., 1977; Ludwig, 1978).

The inability of W19 to utilize any amino-acid except glutamate as sole N source was similar to that found in GOGAT negative mutants

Fig. 3 Growth curves observed with strains 7008 and 7012 growing in RDM-glucose containing 20 mM NH_4Cl, and supplemented with histidine (100 $\mu g\ ml^{-1}$) and tryptophan (100 $\mu g\ ml^{-1}$). The cultures were grown in 250 ml Erlenmeyer flasks containing 50 ml of medium and growth was monitored as described in Section 2. Further histidine (100 $\mu g\ ml^{-1}$) was added to the culture of strain 7012 at the point indicated by the arrow.

■, strain 7008; □, strain 7012 .


absorbance (540 nm)
·90
·50
·40
·30
·20
·10
·05
·02
4
8
12
16
20
24
26
time (h)

of other genera (Nagatani et al., 1971; Berberich, 1972; Brenchley et al., 1973; Funanage et al., 1978; Pahel et al., 1978). The growth of W19 on glutamate was also poor compared to that of other R. trifolii strains. The inability of W19 to grow on most amino-acids as sole nitrogen source could be due to its inability to assimilate the ammonia produced from the catabolism of these amino-acids. However, this does not explain the inability of W19 to grow on nitrogen sources degraded to glutamate because it could grow on glutamate, albeit poorly. Strain W19 must presumably be unable to synthesize the enzymes necessary for the catabolism of the glutamate-yielding amino-acids. Derivatives of W19 able to grow well on glutamate, or amino-acids catabolized directly to glutamate, except arginine, asparagine and glutamine (strain W19 pro L, Table 14) were readily isolated, although the exact frequency at which these derivatives arose could not be estimated because W19 was able to form colonies of visible size, even on media containing purified agar and no nitrogen source (Table 11). Some revertants of GOGAT-negative mutants of enteric bacteria, able to grow on various amino-acids but not low amounts of ammonia as sole N source, were found to synthesize GS constitutively, implicating the GS protein in the regulation of various enzymes that provide the cell with glutamate (Brenchley et al., 1973; Funanage et al., 1978; Pahel et al., 1978). Similar GS constitutive 'revertants' of a GOGAT-negative mutant of K. pneumoniae synthesized nitrogenase in the presence of ammonia (Shanmugam et al., 1975). The growth properties of strain W19 and its large derivatives suggested that an analogous situation might occur in strain W19, i.e. that the synthesis of the enzymes required for the catabolism of amino-acids able to supply the cell with glutamate might be under regulation by glutamine synthetase. Kondorosi et al. (1977) also reported that a GOGAT-negative mutant of R. meliloti only grew poorly on the glutamate-yielding amino-acids,

but grew well on glutamate. However, they made no comment on this, but only estimated the growth from lawns of bacteria. An effect such as that found with W19 could have been masked, and the isolation of derivatives with improved growth on the amino-acids was not reported. Brenchley et al. (1973) found that GOGAT-negative mutants of K. aerogenes contained only low amounts of GS and suggested that this was because the mutants were unable to remove glutamine via GOGAT and therefore had elevated levels of glutamine, resulting in the repression of GS. A similar situation might occur in W19 supplied with glutamate. It is unlikely that W19 contained glutaminase because 'revertants' of W19 able to use ammonia or most amino-acids as sole N source (W19 aut + 1; Table 14) were unable to use glutamine. These revertants were also unable to use asparagine or arginine, other amino-acids normally catabolized to produce glutamate but unable to support the growth of W19 pro L.

A similar pattern to that exhibited by W19 of N source utilization was found in mutants of R. trifolii subsequently found to lack pyruvate carboxylase (pyc) (see Section 6.2.7). However, a tri-carboxylic acid cycle intermediate or glutamine was able to suppress the growth phenotype of these mutants. Neither glutamate nor amino-acids catabolized to form glutamate were able to satisfy the growth requirement, suggesting that these amino-acids were not converted to 2-oxoglutarate in the pyc mutants. This result was surprising because glutamine is normally catabolized by glutaminase to form glutamate. Single-step 'revertants' in which glutamate or any of the amino-acids catabolized to glutamate were able to suppress the growth defect (7049-L, Table 14) arose at a frequency similar to that at which the 'large' derivatives of W19 appeared. It was postulated that the pyc mutants had a glutamine-dependent phenotype, i.e. that glutamine synthetase was inactive in the pyc mutants, and that the mutation that allowed utilization of the other amino-acids suppressed the glutamine-dependency, i.e. that the mutations allowed synthesis

of active GS. This suggests that the synthesis of the enzymes required for the catabolism of the amino-acids is regulated by GS. However, the pyc mutants did not exhibit glutamine-dependency if a tricarboxylic acid cycle intermediate was included in the medium, i.e. addition of succinate or malate must allow the synthesis of active GS.

The following hypothesis was developed to account for the above observations. It is proposed that the activity of GS is normally regulated by the ratio of 2-oxoglutarate to glutamine, as found in enteric bacteria (see Section 1.7.1; also Bender and Magasanik, 1977). The pyc mutants were unable to grow in unsupplemented media because the tricarboxylic acid cycle intermediates used for biosyntheses were not replenished by the anaplerotic enzyme (see Kornberg, 1966). Thus the pyc mutant would be quickly depleted of 2-oxoglutarate. While 2-oxoglutarate was still available, some would be sequestered by GOGAT with the formation of glutamate. This glutamate would be converted to glutamine by GS with the result that the pool of glutamine would be built up when the cell was depleted of 2-oxoglutarate. Thus the 2-oxoglutarate to glutamine ratio in the cell would be very low, with the result that the GS would be highly adenylylated and repressed. The synthesis of the enzymes necessary for the catabolism of the glutamate-yielding amino-acids would, therefore, not be induced, assuming that these enzymes are under the same positive control by GS in R. trifolii as in enteric bacteria. The poor growth on glutamate would be explained if the system for the uptake of glutamate was similarly controlled by GS. Control of amino-acid uptake systems by GS has recently been demonstrated in Salmonella tyhimurium (Funanage et al., 1978). Succinate would be able to activate GS by redressing the 2-oxoglutarate to glutamine ratio. Extracellular glutamine would induce glutaminase and the resulting glutamate would be converted to 2-oxoglutarate by catabolic GDH, once again redressing the 2-oxoglutarate to glutamine ratio. This

assumes that the uptake of glutamine would not be under positive control by GS. The revertants able to utilize the glutamate-yielding amino-acids would possess GS that was insensitive to the 2-oxo-glutarate to glutamine ratio, i.e. the GS would be synthesized constitutively, thus both relieving the glutamine-dependency and allowing induction of the relevant amino-acid catabolizing enzymes.

The finding that the deficiency of two different enzymes (GOGAT and pyruvate carboxylase) in two different strains of R. trifolii resulted in an almost identical phenotype with regard to N source utilization provides support for the hypothesis that in R. trifolii the synthesis of the enzymes required for the catabolism of the glutamate-yielding amino-acids is under the control of GS, which in turn is regulated by the 2-oxoglutarate to glutamine ratio in the cell. However, mutants of W19 presumed to have escaped this regulation (e.g. W19 pro L) were sensitive to repression by ammonia (Table 12) suggesting that ammonia repression was caused by a different mechanism from that causing repression in the GOGAT-negative mutants. However, ammonia did not repress the growth of strains 7000 or 7049-L (data not shown).

In retrospect, it is not surprising that the GS assays failed to reveal any difference in GS activity between rhizobia cultured in the presence of 1 mM NH_4^+ or histidine compared to rhizobia cultured in the presence of 20 mM NH_4^+ (Table 17). This may be due to one or more reasons. The experiments were done with batch cultures wherein the concepts of ammonia or glucose limitation are inapplicable. The growth rate of the Klebsiella strains was always much slower on low ammonia or histidine containing media than on high ammonia containing media, suggesting that the N sources in the former media were growth-rate limiting for Klebsiella. However, Klebsiella has a much shorter doubling time than rhizobia in the media used and it is unlikely that the rhizobia grown in the presence of 1 mM NH_4^+ or histidine were

nitrogen-limited because the addition of extra ammonia did not increase their growth rate. In fact, the presence of 20 mM NH_4Cl caused a pronounced decrease in the growth rate of W19 pro L (see above), but had little or no effect on the growth rate of wild-type bacteria (strain 7000 and isolates VW1 to VW40). It is therefore probable that both 1 mM NH_4^+ and 20 mM NH_4^+ represented ammonia-excess conditions, and therefore the ratio of 2-oxoglutarate toglutamine in the cell may have been similar under both ammonia concentrations. Similarly, no evidence for growth limitation of rhizobia by 0.5 mM or 10 mM histidine was observed. Therefore the results of the GS assays are explicable by assuming that the ratio of 2-oxoglutarate to glutamine in the bacteria was favourable to GS synthesis, with approximately 50% of the enzyme in adenylylated state. The finding of derepressed amounts of histidase, irrespective of the ammonia concentration in the medium, is also consistent with this explanation, as there was always sufficient GS present to activate the transcription of the histidine utilization (hut) genes, assuming that they were sensitive to catabolite repression. The only evidence suggesting that the hut genes were sensitive to catabolite repression was the fact that neither W19 nor the pyc derivatives of 7000 could utilize histidine. It would seem advantageous to the bacteria if the hut genes were under catabolite repression, as neither W19 nor 7000 was able to use histidine as sole carbon source (Primrose, personal communication).

It is also possible that the GS activities measured do not reflect the in vivo situation. The GS transferase assays used were developed for the enzyme from E. coli (Shapiro and Stadtman, 1970), and no attempt was made to find optimal substrate concentrations or pH for the rhizobial enzyme. Recently, however, Bender et al. (1977) found that the biochemical parameters of the transferase activity of GS from K. aerogenes differed in several important respects from those of the E. coli enzyme. The isoactivity point for the adenylylated and

non-adenylylated forms of the enzyme occurred at pH 7.55 in *K. aerogenes* and pH 7.15 in *E. coli* under the conditions used. However, the isoactivity point of the two forms of the enzyme was dependent upon the exact conditions of the assay system; changes in one or more of the components altered the isoactivity point. Also, the activity of the non-adenylylated enzyme from *K. aerogenes* was stimulated by Mg^{2+} but the activity of the enzyme in the absence of Mg^{2+} increased with increasing adenylylation. A further very important point was that both the activity and the adenylylation state of the enzyme changed during harvesting unless special precautions (the addition of hexadexyltrimethylammonium bromide (CTAB) to the medium) were taken before harvesting. This point is also relevant to activities obtained using the biosynthetic assay because this assay, in theory at least, measures only non-adenylylated enzyme. Unfortunately this work (Bender *et al.*, 1977) had not been published at the time the work described above was done, and no special precautions were taken when harvesting the cells. Similarly, the isoactivity point of the two proposed forms of the enzyme was not determined. Therefore the relevance of the GS activities obtained to the *in vivo* situation in the rhizobia must be doubted.

However, the rhizobia did contain GS activity under the conditions tested. Since rhizobia probably do not possess GDH, the GS/GOGAT pathway has to function for ammonia assimilation irrespective of the concentration of ammonia present, and so it would not be surprising if rhizobial GS was not regulated in response to the concentration of ammonia in the environment. The fact that most strains of *R. trifolii* grew equally well in the presence of 20 mM NH_4^+ as they did in the presence of 1 mM NH_4^+ suggests that an adequate amount of GS was synthesized under both conditions. Strain W19 was exceptional in this respect. However, it should be noted that even though *K. aerogenes* regulates its GS in response to ammonia concentration, mutants of *K. aerogenes* lacking GDH have no detectable phenotype, showing that even these bacteria can grow well in the presence of high ammonia concentrations without GDH (Brenchley and Magasanik, 1974).

In summary, it is suggested that the GS of R. trifolii is regulated by the 2-oxoglutarate to glutamine ratio in the cell, but this ratio is relatively constant under most growth conditions, allowing the synthesis of GS. Only under conditions such as those occurring in mutant strains W19 and 7049 or under severe carbon limitation in the chemostat does the ratio become low enough to repress GS. The mechanism of repression of GS may involve adenylylation, but there is no evidence for this. The expression of the enzymes required for the catabolism of various amino-acids is proposed to be under the control of GS, resulting in their synthesis, if inducer is present, under most conditions. These hypotheses allow ample scope for future experimentation. The determinations of the 2-oxoglutarate to glutamine ratios in batch cultures with various nitrogen sources and in chemostat cultures under glucose or nitrogen limitation together with their correlation with amounts and adenylylation of GS are obvious examples.

5.4 Assimilation of ammonia and regulation of nitrogenase in free-living rhizobia and bacteroids: current knowledge

Earlier evidence indicating that GS was present only in repressed form and amounts in bacteroids, and that bacteroids excreted the ammonia produced by nitrogenase was discussed in Section 1.7.2. An understanding of the nature of the regulatory signal(s) which (i) switch on the nif genes, and (ii) allow most of the ammonia produced to be excreted to the plant, is fundamental to the basic understanding of the biology of symbiotic nitrogen fixation. The work published in the last three years aimed at achieving such knowledge is reviewed in this section.

Studies on the control of nitrogenase in rhizobia were aided by the discovery that some Rhizobium strains could fix nitrogen while free-living if cultured under certain conditions, including a supply of fixed nitrogen (see Section 1.4.4). Thus nitrogenase was expressed in Rhizobium under conditions which normally caused its

repression in enteric bacteria. Nitrogenase was induced in the rhizobia (cowpea strain 32 HI) at about the time that the bacteria were entering into the stationary phase of growth, and the bacteria were found to excrete, in the form of ammonia, most of the nitrogen fixed (O'Gara and Shanmugam, 1976; Tubb, 1976). This excretion of ammonia was dependent upon the presence of a fixed nitrogen source (glutamate) in the medium. Glutamate also inhibited the utilization of exogenous ammonia, and it was therefore proposed that the ammonia-assimilatory enzymes of rhizobia were repressed by the presence of one or more amino-acids (O'Gara and Shanmugam, 1976; Tubb, 1976). This was supported by the finding that several species of rhizobia excreted ammonia from histidine when grown on the amino-acid (O'Gara and Shanmugam, 1976) but no enzymatic data in support of the hypothesis was presented. However, Upchurch and Elkan (1978) found that the addition of glutamate to R. japonicum growing on 10 mM ammonia caused a strong repression of GOGAT and increased repression and adenylylation of GS, thus providing further support for a role of glutamate in the control of ammonia-assimilatory enzymes in Rhizobium. However, the strain of R. japonicum used was unusual in that the addition of glutamate caused a dramatic decrease in the growth rate.

Other studies have shown that the dissolved oxygen concentration is critical to the development of nitrogenase activity in free-living rhizobia (see Section 1.5.5). Bergersen and Turner (1976) found that, in chemostat cultures of cowpea rhizobia, nitrogenase activity was unaffected by ammonia or glutamine when growth was severely restricted by limited supply of oxygen. However, when the oxygen supply rate was increased, resulting in an increase in cell-yield but no change in dissolved oxygen concentration, nitrogenase activity was greatly diminished. The amount of nitrogenase

activity at the higher oxygen supply rates was inversely related to the ammonia concentration in the medium, i.e. ammonia caused repression of nitrogenase activity. Also, with higher ammonia concentrations, more stringent oxygen-limitation was required for maximum nitrogenase activity. The relative adenylylation of GS increased with increased rate of oxygen supply in cultures containing excess ammonia, with high nitrogenase activity being invariably associated with low adenylylation of GS. It was suggested that nitrogenase was normally repressed by excess ammonia through adenylylation of GS, but that oxygen-limited growth resulted in a restricted supply of ATP which, in turn, interfered with the adenylylation of GS (Bergersen and Turner, 1976). This implies that under oxygen-limitation ATP is channelled to nitrogenase rather than to GS.

However, in contrast to the above results, Rao et al. (1978) found that the adenylylation of GS increased with decreasing oxygen tension and that at the low oxygen tensions required for nitrogenase synthesis, the GS was highly adenylylated. These experiments were done with batch cultures, presumably in the stationary phase of growth, and grown in medium containing glutamate rather than glutamine or ammonia as used by Bergersen and Turner (1976). Rao et al. (1978) suggested that GS did not have a positive regulatory function in nitrogenase synthesis, but it is difficult to judge the significance of their results because of the ill-defined cultural conditions used. For example, in K. aerogenes and K. pneumoniae the regulation of histidase biosynthesis by GS broke down when the cultures were in the stationary phase of growth (see Section 5.2.5).

Darrow and Knotts (1977) had previously shown that several strains of rhizobia contained two proteins with GS activity as judged by the transferase reaction. The proteins were separable by isoelectric focusing and were termed GSI and GSII. GSI was subject to modification by adenylylation whereas GSII was not.

However, Rao et al. (1978) found that GSII had completely disappeared at the low oxygen tensions required for nitrogenase synthesis, indicating that it also had no positive regulatory role in nitrogenase biosynthesis. Also Ludwig (1978) suggested that GSII may in fact be an asparagine synthetase. However, Ludwig also stated that asparagine synthetase had been found in rhizobia by Scott et al. (1976); in fact the latter authors stated that they were unable to find any asparagine synthetase activity in Rhizobium.

Evidence for a positive role of GS in the control of nitrogenase synthesis was obtained by the isolation of glutamine-dependent (gln) mutants of R. meliloti (Kondorosi et al., 1977), and Rhizobium strain 32HI which nodulates cowpea (Ludwig and Signer, 1977). In both cases, the mutants lacked GS activity and formed only small white nodules which did not reduce acetylene. However, the exact site of the block in the symbiosis was not reported, and it is possible that the symbiosis formed was ineffective for reasons unrelated to the induction of nitrogenase. Nevertheless, the gln mutant of 32HI was also unable to fix nitrogen while free-living (Ludwig and Signer, 1977). In contrast to this, a GOGAT-negative mutant of R. meliloti formed an effective symbiosis, showing that rhizobial GOGAT was not required for any aspect of the symbiosis (Kondorosi et al., 1977).

Kondorosi et al. (1977) also studied ammonia assimilation in free-living R. meliloti. However, they used batch cultures and also assayed GS by the transferase assay using the conditions determined for the E. coli enzyme. Therefore their GS data is open to the criticisms outlined in the previous section. However, they found, in contrast to the results of Upchurch and Elkan (1978), that bacteria cultured with glutamate or glutamine as sole N source contained the highest amount of GS. This amount was reduced by 50% if ammonia was the sole N source and by 90% if casamino-acids were used. In each case the activity of the enzyme was

inhibited approximately 60% by Mg^{2+}, but this inhibition was not removed if the crude extracts were incubated with snake venom phosphodiesterase (SVD), suggesting that the inhibition was not due to adenylylation of the GS. The activity of GS in bacteria grown in the presence of high ammonia concentrations was always adequate to provide the cells with assimilated ammonia. This, in conjunction with the findings that GOGAT was present and biosynthetic GDH absent irrespective of the N source, led to the proposal that R. meliloti assimilated ammonia by the GS/GOGAT pathway, irrespective of the N source present. Thus the results obtained with R. meliloti (Kondorosi et al., 1977) were similar to the results obtained with R. trifolii and reported in this thesis.

Bishop et al. (1976) found that the addition of ammonia to nitrogen-starved R. japonicum caused repression and adenylylation of the GS. However, they also used the transferase assay of Shapiro and Stadtman (1970), but in their studies treatment of the extracts with SVD did result in the relief of Mg^{2+} inhibition, suggesting that adenylylation of GS had, in fact, occurred. They also studied both the effect of the addition of ammonia on GS activity in isolated bacteroids and the effect on GS and nitrogenase activities in the nodules of ammonia addition to nodulated plants. In both cases the addition of ammonia had no effect on the activity of or level of adenylylation of the GS. In contrast to the results of others (see Section 1.7.2), they found that bacteroid GS was only partially adenylylated although the GS was present at a specific activity only 5-20% of that found in nitrogen-starved free-living R. japonicum. Their finding of partial adenylylation could be explained by a decrease in adenylylation during bacteroid extract preparation, similar to that found by Bender et al. (1977) in K. aerogenes. However, Bishop et al. (1976) did find that the addition of ammonia to nodulated plants caused repression of

nitrogenase activity without any apparent effect on plant or bacteroid GS activity or adenylylation, suggesting that the ammonium effect was not mediated by GS. This result was not surprising in view of the evidence indicating that bacteroid GS is at least adenylylated, if not repressed, under nitrogen-fixing conditions (Bishop et al., 1976; Planqué et al., 1977, 1978; see also Section 1.7.2).

Planqué et al. (1978) found an inverse relationship between GS activity, as measured by the transferase assay of Shapiro and Stadtman (1970), and nitrogenase activity during that period of nodule development in which nitrogenase was induced. The GS was always in the highly adenylylated form, as measured by the transferase reaction in the presence of Mg^{2+}. Therefore, these authors suggested that GS did not have a positive regulatory role in the induction of nitrogenase synthesis in R. leguminosarum bacteroids.

Ludwig (1978) studied the control of ammonia assimilation in the strain 32HI which was used by O'Gara and Shanmugam(1976) and Tubb (1976) to show that ammonia was not assimilated and that most of the nitrogen fixed was excreted as ammonia if glutamate was present in the medium. However, Ludwig (1978) seemed to be unaware of this work, because he included glutamate in most media used to culture 32HI but did not cite either O'Gara and Shanmugam (1976) or Tubb (1976). However, Ludwig and Signer (1977) did cite the work of O'Gara and Shanmugam (1976), suggesting that Ludwig (1978) chose to ignore the earlier findings. Ludwig (1978) suggested that 32HI used the GS/GOGAT pathway to assimilate ammonia because GOGAT but not biosynthetic GDH was present in media containing glutamate or glutamate plus ammonia as N source. Also, a mutant supposedly lacking GOGAT was stated to be unable to grow with ammonia as sole N source. However, the amount of GOGAT found in the wild-type 32HI (specific activity 1.2

nmol min^{-1} (mg protein)$^{-1}$) was extremely low, suggesting that the conditions used by Ludwig (1978) were not inducive to GOGAT synthesis. This would be in agreement with the results of Upchurch and Elkan (1978) who found that glutamate repressed GOGAT synthesis in R. japonicum, and it would also provide an explanation for the inability of 32HI to assimilate ammonia when cultured on glutamate (O'Gara and Shanmugam, 1976; Tubb, 1976). GOGAT and GDH activities in bacteria cultured with ammonia as sole N source were not reported by Ludwig (1978). However, growth of 32HI with 0.5 mM ammonia as sole N source was extremely slow, and was further inhibited if the amount of ammonia supplied to the batch cultures was increased.

Ludwig (1978) also studied the control of GS in 32HI, but the significance of his results is difficult to assess. The conditions used for the transferase assay were not clearly defined, but both Shapiro and Stadtman (1970) and Bender et al. (1977) were cited. The assays were done at pH 7.0 which was the pH optimum for adenylylated enzyme. The pH optimum for non-adenylylated enzyme could not be defined because no non-adenylylated GS was found, and therefore the isoactivity point of the two postulated forms of the enzyme could not be determined. However, basically Ludwig found that the highest amount of GS was present in cultures growing with glutamate as sole N source and that the addition of even low amounts (0.1 mM) of ammonia to such cultures (ammonia-shock treatment) resulted in the gradual repression (as assayed by the transferase reaction in the absence of Mg^{2+}) and rapid adenylylation (as assayed by the transferase reaction in the presence of Mg^{2+}) of the GS. However, the amount of GS when assayed by the biosynthetic (forward) reaction was only 1/25 (cf. $\frac{1}{2}$ for GS from enteric bacteria) of that found using the transferase assay (-Mg^{2+}) irrespective of whether the bacteria were grown in media containing glutamate or glutamate plus ammonia as N source. This, he suggested, was evidence that 32HI GS was partially adenylylated under all conditions.

However, when the enzyme purified from bacteria grown with glutamate plus ammonia was treated with SVD, transferase activity in the presence of Mg^{2+} was increased, but if enzyme purified from bacteria grown with glutamate as sole N source was similarly treated, no change in activity was found. This was but one of several paradoxes in his data. Nevertheless, Ludwig (1978), largely on the basis of his ammonia-shock treatment data, proposed that the GS from 32HI was extremely sensitive to repression and inactivation caused by adenylylation of GS in response to even low amounts of ammonia. Since 32HI did not possess assimilatory GDH, it was proposed that ammonia therefore caused 32HI to switch off its ammonia-assimilatory enzymes. If similar control occurred in the bacteroid, this would result in the bacteroid excreting ammonia. It was also proposed that high levels of catalytically-inactive GS might have a regulatory function in nitrogenase synthesis. Such a function had been suggested for highly adenylylated GS from K. aerogenes because mutants of K. aerogenes which constitutively synthesized such GS synthesized histidase if inducer was present (Bender and Magasanik, 1977).

5.5 Assimilation of ammonia in the nodules: current knowledge.

A number of the above studies confirmed that the bacteroid excreted the ammonia produced from nitrogen reduction into the plant fraction of the nodule. Robertson et al. (1975a, b) (see also Section 1.7.2) suggested that the ammonia was converted to glutamine and glutamate by GS and GOGAT of plant origin because the enzymes were induced, in amounts sufficient to account for assimilation of ammonia, in the nodule cytosol in parallel with the induction of nitrogenase in the bacteroid. However, asparagine rather than glutamate or glutamine was the predominant amino-acid transported in the plant xylem (Robertson et al., 1975b). This was explained by the finding that a glutamine-dependent asparagine

synthetase was also induced in the nodule cytosol in parallel with nitrogenase (Scott et al., 1976). The enzyme catalyzed the reaction:

aspartate + glutamine + ATP → asparagine + glutamate + AMP + PPi

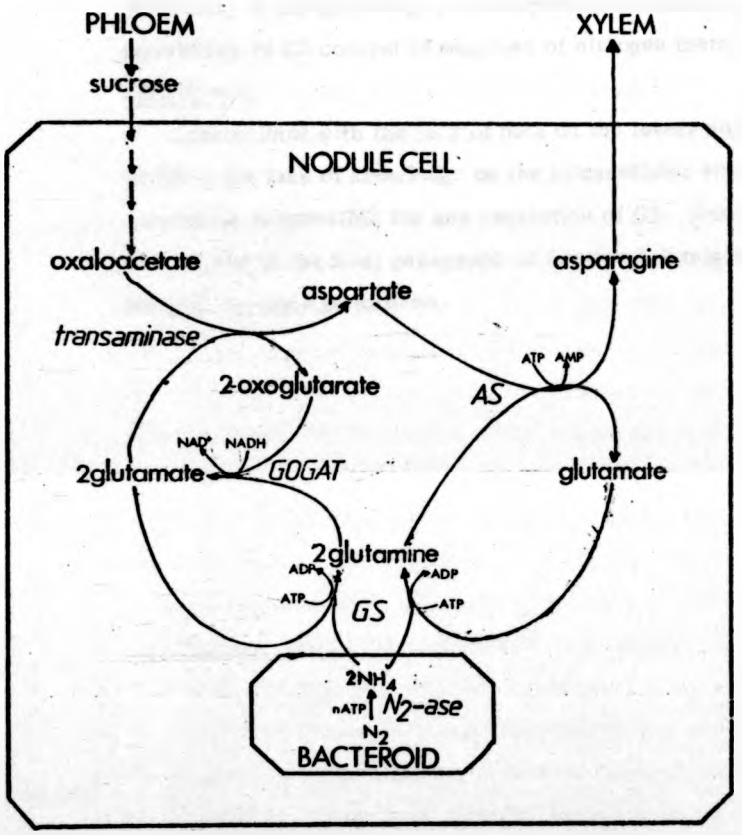
Therefore Scott et al. (1976) proposed the scheme outlined in Fig. 4 for the assimilation of ammonia into asparagine, involving enzymes located in the plant-fraction of the nodule. The net result of the reactions would be the synthesis of one mole of asparagine from one mole of oxaloacetate and 2 mole of ammonia, with one mole of NADH and 3 mole of ATP being consumed. This scheme has not been challenged since its proposal. Christeller et al. (1977) showed that phosphoenolpyruvate carboxylase was also induced in the nodule cytosol in parallel with nitrogenase, and suggested that the enzyme may provide all of the oxaloacetate required for the transamination reaction.

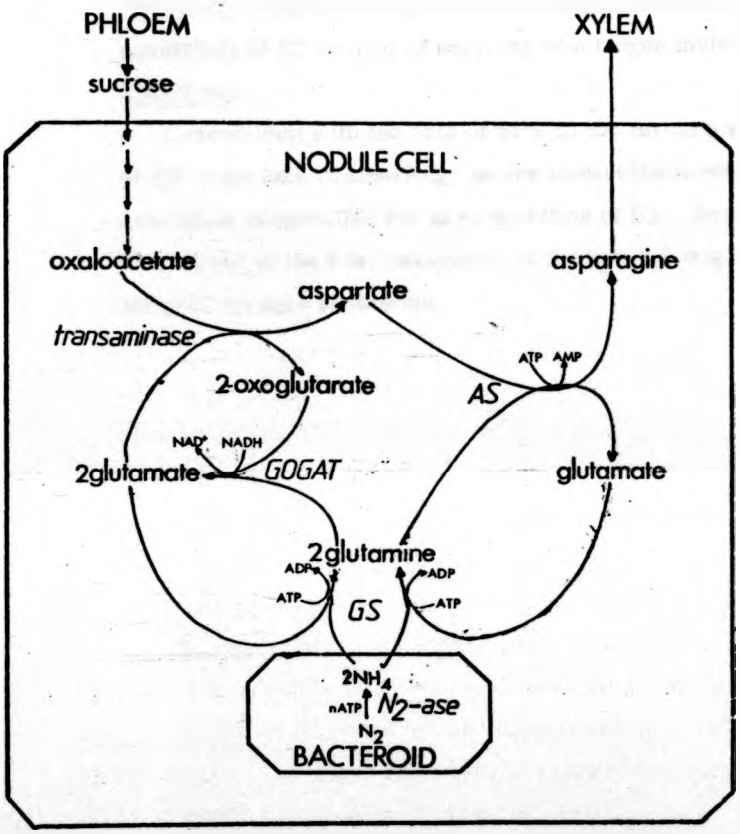
Thus the assimilation of ammonia is achieved by the co-ordinated induction of GS, GOGAT, asparagine synthetase and phosphoenol-pyruvate carboxylase in the nodule cytosol at the time, that nitrogenase synthesis is induced in the bacteroids. The induction of leghaemoglobin also follows similar kinetics. The mechanism(s) underlying the co-ordinated induction of the synthesis of these proteins is unknown.

5.6 Conclusions

Although much effort has been devoted to studying rhizobial GS and GOGAT, definitive studies on nitrogen assimilation in Rhizobium have still not been done. Much of the work done is open to the criticisms mentioned in Sections 5.3 and 5.4, and so especially lacking are studies on the levels and regulatory properties of GS under conditions of nitrogen or carbon limitation. Conditions of nitrogen limitation presumably prevail during the period of nodule development before nitrogenase is induced, because the plant is nitrogen-stressed. Therefore the possibility that GS may play a

Fig. 4 The pathway of ammonia assimilation in a mature nodule cell (from Scott et al., 1976).
Abbreviations: AS, asparagine synthetase; GOGAT, glutamate synthase; GS, glutamine synthetase; N_2-ase, nitrogenase.


PHLOEM
XYLEM
sucrose
NODULE CELL
oxaloacetate
asparagine
aspartate
transaminase
ATP AMP
2-oxoglutarate
AS
NAD+ NADH
2glutamate
GOGAT
glutamate
2glutamine
ADP
ATP
ADP
ATP
GS
2NH4
nATP
N2-ase
N2
BACTEROID


PHLOEM
XYLEM
sucrose
NODULE CELL
oxaloacetate
asparagine
aspartate
transaminase
ATP AMP
2-oxoglutarate
AS
NAD+ NADH
2glutamate
GOGAT
glutamate
2glutamine
ADP
ATP
GS
ADP
ATP
2NH4
nATP
N2-ase
N2
BACTEROID

positive role as a trigger for nitrogenase induction has not yet been discounted. The studies on the growth of strains W19 and 7049 are, to my knowledge, the only ones to demonstrate the possibility of GS control of enzymes of nitrogen metabolism in Rhizobium.

Concomitant with the lack of data on the levels and activity of GS is the lack of knowledge on the intracellular environmental conditions responsible for any regulation of GS. Experiments such as outlined in the final paragraph of Section 5.3 might provide insight into such conditions.

CHAPTER 6

STUDIES ON CARBOHYDRATE METABOLISM* IN R. trifolii

6.1 Introduction

The pathways of central carbohydrate catabolism in rhizobia have been only poorly studied (see Section 1.6). However, carbohydrate utilization has been proposed as a taxonomic aid to differentiate between fast- and slow-growing rhizobia (Graham, 1964). In general, slow-growing strains did not grow on maltose, trehalose, dulcitol, sucrose, lactose, rhamnose or raffinose, whereas fast-growing strains grew on most carbohydrates. L-Arabinose and xylose were found to be the best carbon sources for slow-growing strains (Graham, 1964; Vincent, 1970) while mannitol was often used for fast-growing strains (Fred et al., 1932; Vincent, 1970). The pathways used for catabolizing some of these preferred carbon sources have been investigated in a few species.

The polyols mannitol, arabitol and sorbitol were oxidized by inducible NAD^+-linked dehydrogenases in R. meliloti, R. leguminosarum and R. trifolii (Martinez-de Drets and Arias, 1970). Growth of R. meliloti on mannitol induced dehydrogenases active with all three polyols. Evidence was presented that mannitol and arabitol were oxidized by one enzyme, arabitol dehydrogenase, whereas sorbitol was oxidized by a separate enzyme, sorbitol dehydrogenase. Arabitol was oxidized to xylulose and mannitol and sorbitol to fructose. Growth of R. meliloti on a polyol plus either glucose or fructose resulted in a 70-80% repression of the polyol dehydrogenase activities (Martinez-de Drets and Arias, 1970). No evidence for polyol-phosphate dehydrogenase activity was found suggesting that the oxidation preceded the phosphorylation of the polyols. This infers that mannitol was taken up without concomitant phosphorylation by the bacteria.

* All sugars are of the D-configuration unless otherwise stated.

Similar results were found for mannitol metabolism in R. japonicum (Kuykendall and Elkan, 1977). However, in this case, the inclusion of glucose plus mannitol in the medium resulted in only a 28% repression of the amount of mannitol dehydrogenase. Also, the ratio of activity obtained with mannitol as substrate to that obtained with arabitol as substrate was 2.0, whereas in R. meliloti it was 0.2.

In R. japonicum, L-arabinose was metabolized by an oxidative pathway analogous to the ED pathway of glucose metabolism (Pedrosa and Zancan, 1974). The pathway found was as follows:- L-arabinose $\xrightarrow{1}$ L-arabinolactone $\xrightarrow{2}$ L-arabonate $\xrightarrow{3}$ 2-oxo-3-deoxyarabonate $\xrightarrow{4}$ pyruvate + glycoaldehyde.

Step 1 was catalyzed by L-arabinose dehydrogenase which was also reported to show NAD^+ or $NADP^+$-linked D-fucose and D-galactose dehydrogenase activities, although specific activities were not given. The pathway was expressed constitutively in the strain of R. japonicum used, and was similar to that found in an unidentified Pseudomonas species (Dahms and Anderson, 1969).

Lactose metabolism in R. meliloti was studied by Niel et al. (1977). They found considerable β-galactosidase activity in the absence of inducer, but the amount of activity was increased five-fold by growth in media containing lactose as sole C source. A mutant which grew slowly on lactose lacked the inducible activity, leading to the suggestion that R. meliloti possessed two enzymes, one of which was inducible, with β-galactosidase activity (Niel et al., 1977). The cause of the inability of slow-growing species to grow on lactose has not been investigated. However, slow-growing species were unable to grow on sucrose because they lacked invertase which fast-growing species possessed (Martinez-de Drets et al., 1974).

However, as described earlier, a more basic difference between fast- and slow-growing species was the possession of $NADP^+$-linked 6PG dehydrogenase, indicating that the former species possessed the oxidative PP pathway which was lacking in the latter species. It was

thus thought that the slow-growing species catabolized hexoses solely by the ED pathway, whereas the fast-growing species were able to use the EMP and PP pathways as well (see Section 1.6.3; also review by Vincent, 1977). Almost nothing was known about carbohydrate metabolism in the bacteroid prior to this study, although hexoses were thought to be the main substrates used (see Section 1.6.2; also review by Bergersen, 1977b).

The aim of this section of work was to investigate carbohydrate metabolism in R. trifolii, and, by the isolation of mutants defective in carbohydrate metabolism, to delineate the physiologically important pathways, both in free-living rhizobia and in the bacteroid.

6.2 Results

6.2.1 Enzymological studies on wild-type strain 7000

A number of enzymes involved in carbohydrate metabolism were measured in crude extracts prepared from strain 7000 grown with glucose as sole carbon source (Table 20). Similar values were obtained when glycerol was sole carbon source indicating that none of the enzymes were induced specifically by hexoses. Growth on fructose resulted in an increased amount of fructokinase (196 nmol min^{-1} (mg protein)$^{-1}$) but other enzyme activities remained the same.

In no case was it possible to demonstrate phosphofructokinase activity in cell-free extracts. Considerable effort was directed towards finding this enzyme because it is a key enzyme of the EMP pathway whose presence had previously been reported in R. trifolii (Katznelson and Zagallo, 1957; Vincent, 1977). The buffers used for the preparation of the extracts are listed in Table 21. The supernatant fraction of the cell-free extracts still contained NADH-oxidase activity (up to 60 nmol min^{-1} (mg protein)$^{-1}$), even after extended centrifugation at 38000 g, which may have masked low amounts of phosphofructokinase activity. Various treatments were applied to the cell-free extracts in an attempt to remove the NADH-oxidase activity (Table 22),

Table 20 Activities of some enzymes of carbohydrate metabolism in R. trifolii strain 7000

Bacteria were grown in RDM containing 0.4% (w/v) glucose. Extracts were prepared and enzymes assayed as described in Materials and Methods (Section 2).

Enzyme	Activity nmol min^{-1} (mg protein)$^{-1}$
Glucokinase	178
Fructokinase	107
Glucosephosphate isomerase	1200
Glucose-6-phosphate dehydrogenase	214
6-Phosphogluconate dehydrogenase	116
Fructose diphosphate aldolase	10
Phosphofructokinase	0
Galactose dehydrogenase ($NADP^+$-linked)	190
Galactose dehydrogenase (NAD^+-linked)	200
'ED enzyme'*	108
Isocitrate dehydrogenase	885
Malate dehydrogenase	920

* 'ED enzyme' represents the activity of the Entner - Doudoroff pathway as measured by the production of pyruvate from 6-phosphogluconate.

Table 21 Conditions used for the preparation of extracts of strain 7000 to be assayed for phosphofructokinase activity*

Buffer (20 mM)	Dithioerythritol (1mM)	$MgCl_2$ (10 mM)
HEPES, pH 7.6	-	-
HEPES, pH 7.6	+	-
HEPES, pH 7.6	-	+
HEPES, pH 7.6	+	+
potassium phosphate, pH 7.6	-	-
potassium phosphate, pH 7.6	-	+
Tris-HCl, pH 8.0	-	-
Tris-HCl, pH 8.0	+	+

Key: -, absent; +, present in buffer.

*In all cases, phosphofructokinase was assayed as described by Ling et al. (1966).

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but in no case was fructose 6-phosphate-dependent oxidation of NADH in the coupled assay observed. The routine conditions used for the phosphofructokinase assay were as described by Ling et al. (1966). However, the assay was also done, using cell-free extracts prepared as described in Section 2.6 in 50 mM potassium phosphate buffers covering the pH range 6.0 to 8.0 in gradations of 0.2 pH units, and in 50 mM Tris-HCl buffers covering the pH range 8.0 - 9.0, also in 0.2 pH unit gradations. Since ATP is an allosteric inhibitor of phosphofructokinase (see Sols and Salas, 1966), four final concentrations of ATP (0.01 mM, 0.05 mM, 0.1 mM, 2.0 mM) were used in theassays at each pH. The addition of AMP, which reverses the inhibition of phosphofructokinase by ATP (Sols and Salas, 1966) also failed to reveal phosphofructokinase activity. It was therefore considered unlikely that R. trifolii 7000 contained phosphofructokinase activity. This result, coupled with the low specific activity of fructose diphosphate aldolase (the assay of which was also subject to interference by the NADH oxidase activity in the extracts), indicated

Table 22 Effect of various treatments on NADH oxidase activity in extracts of R. trifolii 7000

The bacterial extract was prepared and NADH oxidase assayed as described in Sections 2.6 and 2.9.5.

Treatment	% reduction in NADH oxidase activity
Addition of 3 mM KCN	50
Anaerobic conditions*	30
Ammonium sulphate precipitation**	100
Heat (50° C, 5 min)	100

*Anaerobic conditions were obtained by bubbling N_2 through a cuvette, capped with a rubber septum ('Suba - Seal', Wm. Freeman & Co., Barnsley, Yorkshire) for 10 min. Final reagents (also treated with N_2) were added with a syringe.

**The extract was brought to 40% saturation with ammonium sulphate at 4° C, stirred slowly for 10 min, and then centrifuged at 10,000 g for 20 min. The supernatant was then dialyzed against 20 mM HEPES buffer (pH 7.6) for 4 h and then used for the assay (Canovas and Kornberg, 1969).

that the EMP pathway was unlikely to be physiologically significant in R. trifolii 7000. However, the failure to detect phosphofructokinase activity does not rule out its existence. For example, it was impossible to assay the particulate fraction of the cell-free extracts for phosphofructokinase, and other enzymes whose assay was based on NADH oxidation, because of the large amount (up to 1500 nmol min^{-1} (mg protein)$^{-1}$) of NADH oxidase activity which this fraction contained.

The presence of the ED pathway was shown by the production of pyruvate from 6PG in the absence of $NADP^+$. Maximum production of pyruvate by extracts of strain 7000 under the conditions used occurred within 30 min (see Fig. 9, Section 6.2.6) giving a specific activity of 108 nmol min^{-1} (mg protein)$^{-1}$, approximately four-fold the level of 'ED enzyme' found in R. trifolii by Martinez-de Drets and Arias (1972). These workers, however, ran their assays for 1 h and thus may have seriously underestimated the specific activity. A maximum of 3 μmoles of pyruvate was accumulated under the conditions used from the 5 μmoles of 6PG initially present and the addition of sodium arsenite (6 μmoles) which inhibits the oxidative decarboxylation of pyruvate, had no effect on this amount.

A high activity of 6PG dehydrogenase, the key enzyme of the PP pathway, was also found, as were various enzymes of the tricarboxylic acid cycle, indicating the potential for these pathways to operate in R. trifolii (Table 20).

Both NAD^+- and $NADP^+$-linked galactose dehydrogenase activities were also found (Table 20). The amount of these activities did not vary irrespective of the carbon source used for the growth of the bacteria.

6.2.2 Isolation of mutant strains

The aim of this section of work was to isolate mutants useful for studying (i) central carbohydrate catabolism in R. trifolii 7000, and

(ii) the role, if any, of hexose catabolism by the bacteroid in nodule function. The mutant strains isolated and used are listed in Table 23 . The mutageneses and enrichments were done as outlined in Materials and Methods (Section 2.4) and the media and the number of ampicillin enrichment cycles used for the isolation of the mutant strains are described in Table 24.

The procedure used to isolate strains 7009, 7013, 7015 and 7062 was originally designed to yield amino-acid auxotrophs. However, the enriched cultures in GSYC took 6 days to become turbid and approximately 50% of the populations displayed a glucose-negative phenotype. This result was particularly surprising in view of glucose being the major carbon source in the GSYC medium used for growth following ampicillin enrichment. Over 400 colonies from each of the two enriched cultures were tested, but no amino-acid auxotrophs were found in these experiments. Twenty of the glucose-negative mutants from each enrichment were further characterized. Strains 7009 and 7013 were then selected for further study because of their 'tight' phenotype and low reversion frequency. Strain 7062 was chosen because it was slightly leaky and therefore of independent origin from strain 7009. Strain 7015 was also chosen for further study because, in contrast to the other 39 mutants tested, it was unable to grow on mannitol in addition to glucose. A subsequent attempt to repeat this experiment, i.e. to isolate glucose-negative mutants using GSYC as non-selective medium, was unsuccessful, although auxotrophs were isolated. Thus the basis of the selection for the glucose-negative phenotype in the original experiments is not understood.

Strains 7028 and 7039 were isolated on the basis of their fructose-negative phenotype, and were the only fructose-negative mutants found from over 1000 microcolonies picked from RDM-fructose plates solidified with Difco-Bacto agar.

Table 23 Mutants defective in carbohydrate metabolism derived from R. trifolii 7000

Strain	Characteristics*	Source
7008	trp-4	NTG mutagenesis of 7000 (see Section 3.2.2)
7009	trp-4 glk-4	NTG mutagenesis of 7008 (this Section)
7012	his-6	NTG mutagenesis of 7000 (see Section 3.2.2)
7013	his-6 glk-7	NTG mutagenesis of 7012 (this Section)
7014	glk-4	Trp^+ revertant of 7009 (this Section)
7015	his-6 glk-1 (Sam^-)	NTG mutagenesis of 7012 (this Section)
7028	his-6 edp-1	NTG mutagenesis of 7012 (this Section)
7039	his-6 glk-7 fup-1	NTG mutagenesis of 7013 (this Section)
7049	pyc-2	NTG mutagenesis of 7000 (see Section 5.2.3)
7056	pyc-18	NTG mutagenesis of 7000 (see Section 5.2.3)
7062	trp-4 glk-2	NTG mutagenesis of 7008 (this Section)

* edp, Entner-Doudoroff pathway; fup, fructose uptake; glk, glucokinase; his, histidine; pyc, pyruvate carboxylase; Sam^-, sorbitol, arabitol and mannitol negative; trp, tryptophan.

Table 24 Media and number of ampicillin enrichment cycles used to isolate mutant strains

Carbon sources (0.4% w/v) and amino acids (100 μg ml^{-1}) were added to RDM* as stated.

Strain	Non-selective medium	Selective medium	No. of ampicillin enrichment cycles
7009 7062	GSYC**	RDM-glucose tryptophan	1
7013 7015	GSYC	RDM-glucose histidine	1
7028	RDM-inositol histidine	RDM-fructose histidine	3
7039	RDM-inositol histidine	RDM-fructose histidine	3
7049	RDM-glucose glutamine, glutamate	RDM-glucose	3
7056	GSYC		0

*RDM: defined medium

** GSYC: glucose-salts-yeast extract-casein hydrolyzate medium

Strain 7049 was one of many succinate-dependent mutants found in six independent experiments (see Section 5.2.3). Eight other succinate-dependent mutants from the same series of experiments were tested and found to display an identical phenotype to strain 7049. Strain 7056 was isolated from a separate experiment designed to isolate non-specific auxotrophs (Section 5.2.3).

6.2.3 Growth of mutant strains on different carbon sources

The growth of these mutant strains on some carbon sources is shown in Table 25 . Neither wild-type nor mutant strains were able to grow on glucose 6-phosphate, sodium gluconate or sodium glucuronate. Growth on succinate was obtained only on solid media. Doubling times were calculated for some of the strains in RDM containing some of the carbon sources. These are given in Table 26.

6.2.4 Characterization of strains 7009, 7013, 7015 and 7062

These four strains, originally found to be glucose-negative, also had impaired growth on cellobiose, maltose, trehalose, maltitol, lactose, lactulose, sucrose, raffinose and dulcitol. In addition, strain 7015 was unable to grow on arabitol, mannitol and sorbitol (Table 25). Strain 7062 was slightly leaky compared to strain 7009 and when grown on YGA plates containing bromothymol blue (20 mg l^{-1}) produced an acid reaction, whereas strain 7009 produced an alkaline reaction, presumably caused by ammonia released when amino-acids in the yeast extract were utilized as carbon source. Strain 7009 was used for most of the following work because the histidine auxotrophy of strains 7013 and 7015 caused a cessation of growth at lower cell densities than those obtained with strain 7009 (see Section 5.2.5, Fig. 3).

Representative growth curves obtained with strains 7008 and 7009 are given in Fig. 5. Growth of strain 7009 was diauxic on certain carbon sources (Fig. 5, a, b, c, d, e, g), the initial growth in broth presumably being due to the utilization of storage carbohydrate by the

Table 25 Growth of mutant strains on different carbon sources*

Growth was determined by streaking single colonies on RDM plates containing the specified carbon source and (except for strains 7049 and 7056) histidine and tryptophan, and solidified with Difco Noble agar. The amount of growth was recorded after incubation of the plates at 28° C for 5 days.

	7000	7009	7062	7013	7015	7039	7028	7017	7049	7056
Glucose	+++	-	-	-	-	-	-	+++	-	-
Fructose	+++	+++	+++	+++	+++	-	±	+++	-	
Galactose	+++	+++	+++	+++	+++	+++	+++		-	-
Mannose	+++	+++	+++	+++	+++		±			
Cellobiose	+++	-	-	-						
Maltose	+++	-	-	-	-					
Trehalose	+++	-	-	-						
Lactose	++	-	-	-	-	-	+++		-	
Lactulose	++	-		±						
Sucrose	+++	-	±	+	±	-	+		-	
Melibiose	+++	++	++	++	++					
Raffinose	++	-		-						
Rhamnose	++	-		-						
Maltitol	++	±		±	±					
Dulcitol	++	-	-	-	-			++		
Inositol	+++	+++	+++	+++	+++	+++	+++	+++		
Mannitol	+++	+++	+++	+++	-	+++	±	-	-	
Sorbitol	+++	+++	+++	+++	-	+++	±	-	-	
Arabitol	+++	+++	+++	+++	-		+++	-		
Ribitol	++	++	++				+++	++		
Xylitol	+	+	+	+	+			+		
L-arabinose	+++	+++		+++	+++		+++			
Ribose	+++	+++		+++	+++	+++	+++		-	
Glucosamine	++	++	++							
Glycerol	+++	+++	+++	+++	+++	+++	+++		-	
Glucose + 1 mM succinate	+++	-	-	-	-	-	-		+++	+++
Succinate	++	++				++	++		++	-

*+++: single colonies > 2.0 mm after 5 days.
++: single colonies 1.0-2.0 mm after 5 days.
+ : single colonies 0.4-1.0 mm after 5 days.
±: single colonies 0.1-0.4 mm after 5 days.
-: single colonies < 0.1 mm after 5 days.

Table 26 Doubling times of various strains in RDM-glucose broths containing various carbon sources

Doubling times (h) were determined as described in Materials and Methods (Section 2.8) and are the average of two determinations. The broths were supplemented with histidine or tryptophan as required.

Carbon source	Strain						
	7008	7009	7012	7013	7014	7028	7039
Glucose	5.1	>50.0	3.5	>50.0	>50.0	>50.0	
Glucose & Galactose	4.1	4.1					
Fructose			3.6			14.0	16.0
Maltose	5.8	>50.0					
Sucrose	6.2	27.2	4.0	14.1	27.2	7.6	
Sorbitol			3.4			9.2	
Mannitol	5.2	4.6	3.5			10.0	
Dulcitol	9.0	16.4					
Galactose			3.4			3.5	
Lactose	6.0	39.0	4.3	30.0			
Lactulose	8.0	22.8					
Ribitol	6.4	6.2					

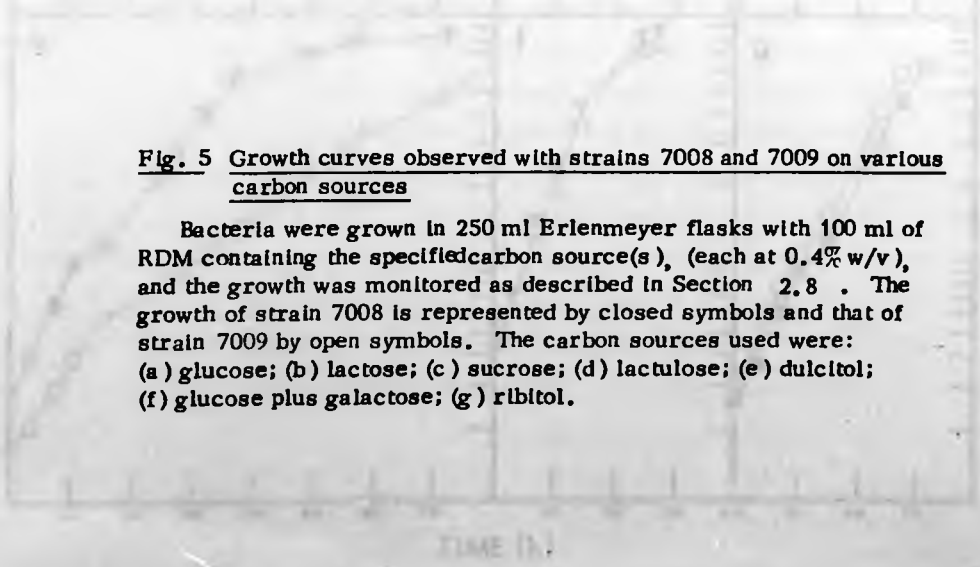
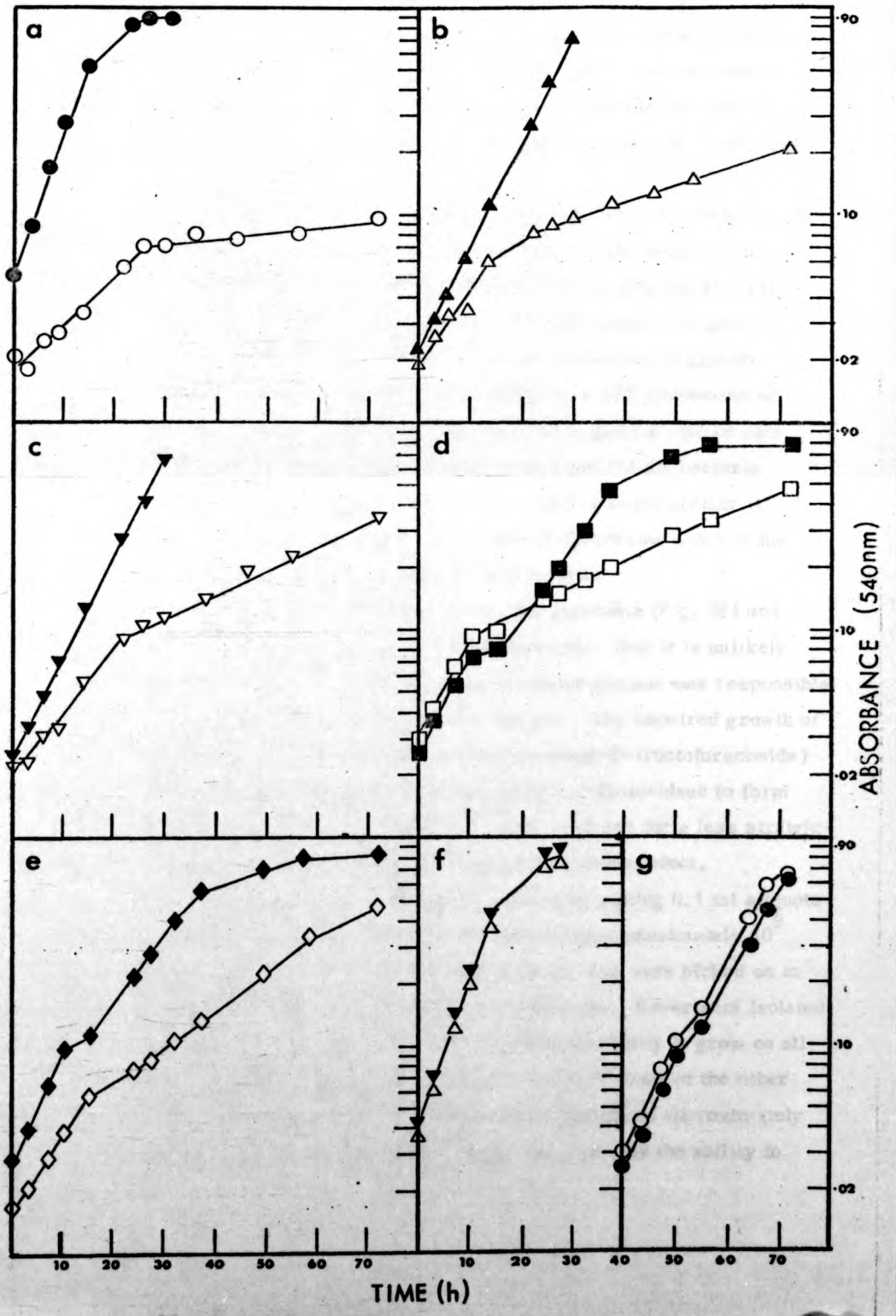


Fig. 5 Growth curves observed with strains 7008 and 7009 on various carbon sources

Bacteria were grown in 250 ml Erlenmeyer flasks with 100 ml of RDM containing the specifiedcarbon source(s), (each at 0.4% w/v), and the growth was monitored as described in Section 2.8 . The growth of strain 7008 is represented by closed symbols and that of strain 7009 by open symbols. The carbon sources used were: (a) glucose; (b) lactose; (c) sucrose; (d) lactulose; (e) dulcitol; (f) glucose plus galactose; (g) ribitol.


a
b
c
d
e
f
g
·90
·10
·02
ABSORBANCE (540nm)
10
20
30
40
50
60
70
TIME (h)

mutant strain. This growth pattern was also observed with strain 7008 when it was grown on carbon sources which were only poorly utilized (Fig. 5, d, e, g). Growth rates were calculated from the linear part of the curve obtained after the initial growth phase, and are given in Table 26.

Strain 7009 could take up glucose at a similar initial rate to wild-type cells (Fig. 6, a, b). The data in Fig. 6a also show that non-induced cells of strain 7008 were able to take up glucose at a rate approximately 50% of that observed with cells grown with glucose as sole carbon source. The presence of inositol in the growth medium, along with the glucose, resulted in a 32% repression of maximal glucose uptake rate. The fall-off in glucose uptake rate with time in strain 7009 (Fig. 6b) was as expected for bacteria accumulating glucose because of their inability to metabolize it. Therefore the pleiotropic growth defect of the strains was not due to a defect in the uptake system(s) for glucose.

Growth of strain 7009 on glucose plus galactose (Fig. 5f) and on glucose plus fructose was at wild-type rate; thus it is unlikely that general growth inhibition by accumulated glucose was responsible for the growth defect on sucrose or lactose. The impaired growth of strain 7009 on lactulose (β-D-galactopyranosyl-D-fructofuranoside) (Fig. 5d), which was presumably split by β-galactosidase to form fructose plus galactose, provided further evidence for a less straightforward cause for the observed pleiotropic growth defect.

Revertants of strain 7009 were isolated by plating 0.1 ml aliquots of a mannitol-grown washed culture containing approximately 10^9 bacteria ml^{-1} on to the appropriate medium, and were picked on to the same medium before testing their phenotype. Revertants isolated on glucose (frequency 10^{-6}-10^{-7}) regained the ability to grow on all carbon sources, whereas revertants isolated on those of the other sugars which were not polymers of glucose produced approximately equal numbers of sugar-specific (i.e. regained only the ability to

Fig. 6 Uptake of glucose by strains 7008, 7009 and 7039

Bacteria were grown in RDM containing 100 $\mu g\ ml^{-1}$ histidine, 100 $\mu g\ ml^{-1}$ tryptophan and the specified carbon sources, and were harvested and assayed for glucose uptake as described in Section 2.11.
(a) Strain 7008 grown with: ♦, 0.4% (w/v) glucose as carbon source;
□, 0.4% (w/v) glucose plus 0.4% (w/v) inositol as carbon source;
▲, 0.4% (w/v) inositol as carbon source.
(b) Strain 7009 grown with: □, 0.4% (w/v) glucose plus 0.4% (w/v) inositol as carbon source;
Strain 7039 grown with: ●, 0.4% (w/v) glucose plus 0.4% (w/v) inositol as carbon source.

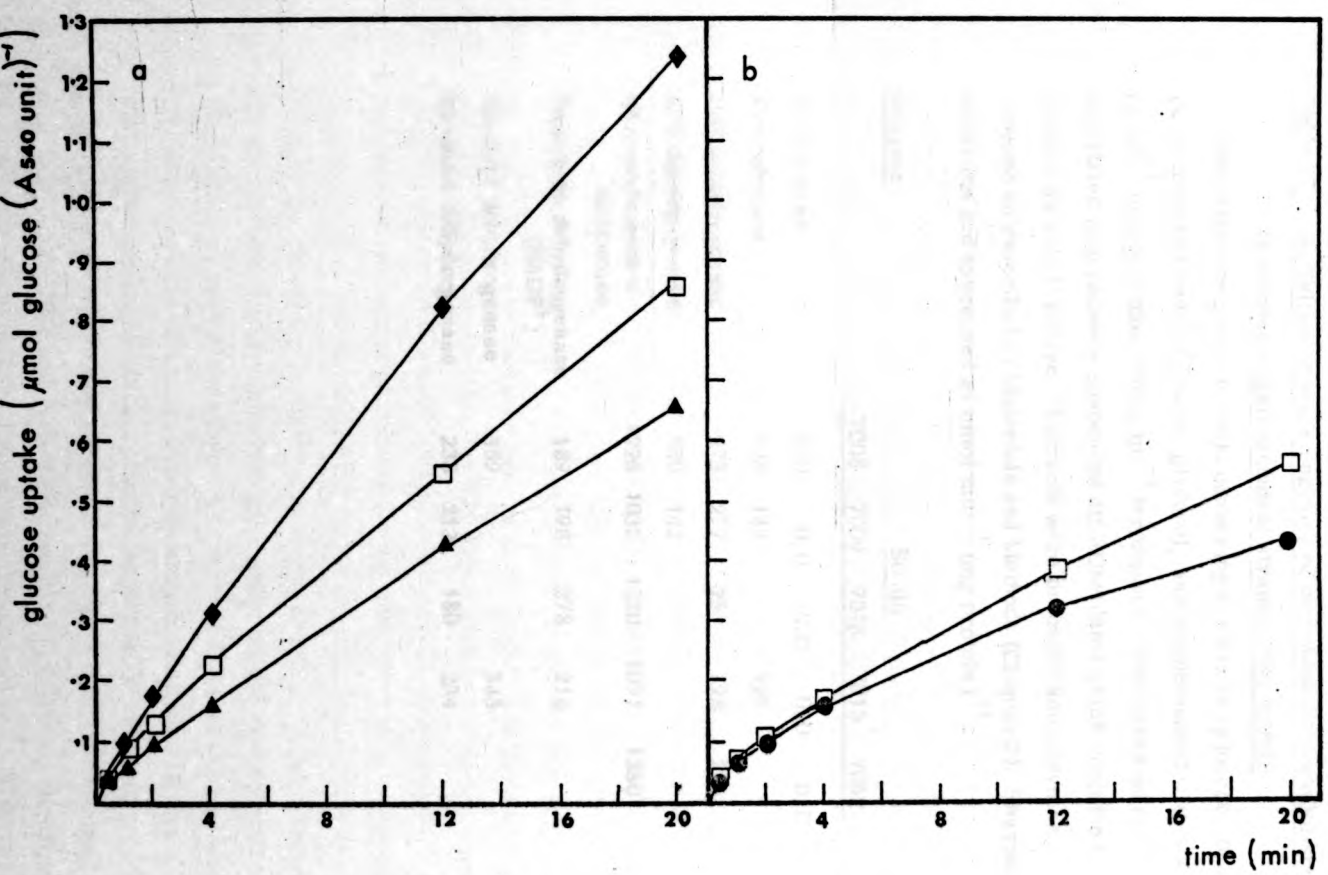

glucose uptake (μmol glucose (A540 unit)⁻¹)
1·3
1·2
1·1
1·0
·9
·8
·7
·6
·5
·4
·3
·2
·1
a
b
4
8
12
16
20
time (min)

Table 27 Activities of some enzymes of carbohydrate metabolism in glucose-negative mutant strains of R. trifolii

Bacteria were grown in RDM containing 0.4% (w/v) glucose, 0.4% (w/v) mannitol and 0.4% (w/v) glycerol, and supplemented with 100 $\mu g\ ml^{-1}$ histidine plus 100 $\mu g\ ml^{-1}$ tryptophan. The flasks were inoculated with bacteria suspended off RDM-plates which contained inositol as sole C source. Extracts were prepared and enzymes assayed as described in Materials and Methods (Chapter 2). Enzyme activities are expressed as nmol min^{-1} (mg protein)$^{-1}$.

Enzyme	Strain				
	7008	7009	7013	7015	7062
Glucokinase	200	0.0	0.0	0.0	0.0
Fructokinase	123	142		123	
G6P dehydrogenase	273	277	251	198	225
6PG dehydrogenase	130	142			
Glucosephosphate isomerase	1226	1032	1320	1091	1230
Galactose dehydrogenase ($NADP^+$)	189	198	278	214	
Arabitol dehydrogenase	359			343	
Mannitol dehydrogenase	235	212	180	234	

grow upon the sugar upon which they were isolated) and 'fully-reverted' phenotypes. These results suggested that the defect causing the inability to grow on glucose was the primary effector of the pleiotropic growth phenotype and also indicated that the phenotype was caused by a single point mutation. Further studies on the nature of the defective growth of the strains on the sugars other than glucose are described in Section 7.2.1.

All four glucose-negative strains (7009, 7013, 7015, 7062) lacked detectable amounts of glucokinase, but possessed glucosephosphate isomerase and G6P dehydrogenase (Table 27). Strain 7009 was also examined for the presence of the other enzymes of hexose metabolism listed in Table 20 and all were found. All of the strains, including strain 7015 which was unable to grow on mannitol, sorbitol or arabitol, possessed mannitol and arabitol dehydrogenase activities. A single enzyme is responsible for these activities in R. meliloti (Martinez-de Drets and Arias, 1970) and probably also in R. trifolii (Primrose, personal communication). All strains, including 7015, also possessed fructokinase which is also necessary for mannitol and sorbitol metabolism in Rhizobium (Martinez-de Drets and Arias, 1970). Further studies on strain 7015 are reported in Section 7.2.1.2.

Thus the results indicated that the primary defect in the pleiotropic glucose-negative strains was the loss of glucokinase. Four glucose-positive revertants of strain 7009 originally tested all regained comparable amounts of glucokinase to the wild-type strain, suggesting that glucokinase was necessary for the phosphorylation of glucose in R. trifolii. However, in very recent work, aimed at finding whether the glucokinase-negative (genotype glk) mutation was in a structural or regulatory gene for the enzyme (see Section 7.2.1.4) it was found that approximately 50% of the glucose-positive revertants of strain 7009 did not regain significant glucokinase activity. Moreover, none of the twenty glucose-positive revertants (frequency approximately 10^{-8}) of strains 7013 and 7015 tested regained significant glucokinase activity (specific activity < 10 nmol min^{-1} (mg protein)$^{-1}$), although most, but by no means all, were able to grow on glucose at a similar

grow upon the sugar upon which they were isolated) and 'fully-reverted' phenotypes. These results suggested that the defect causing the inability to grow on glucose was the primary effector of the pleiotropic growth phenotype and also indicated that the phenotype was caused by a single point mutation. Further studies on the nature of the defective growth of the strains on the sugars other than glucose are described in Section 7.2.1.

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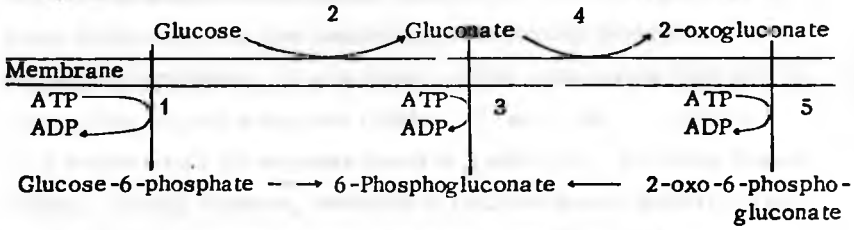
rate to wild-type. Therefore, these revertants must presumably have gained another pathway for forming glucose 6-phosphate, unless the glucokinase formed in them was exceptionally unstable and destroyed during extract preparation. Attempts were made to find pathways such as those found in Pseudomonas aeruginosa (see, for example, Whiting et al., 1976a; Fig. 7) in glucose-positive glucokinase-negative revertants grown with glucose as sole carbon source. Both the particulate and the supernatant fractions of the cell-free extracts

Fig. 7 Pathways of glucose metabolism leading to 6-phosphogluconate in Pseudomonas aeruginosa (adapted from Whiting et al., 1976a).



Enzymes:
1. Glucokinase
2. Glucose dehydrogenase (EC 1.1.1.47)
3. Gluconokinase (EC 2.7.1.12)
4. Gluconate dehydrogenase (EC 1.1.99.3)
5. 2-oxogluconate kinase

prepared from two such revertants (one derived from 7009 and the other from 7013) were assayed for glucose dehydrogenase, gluconate dehydrogenase and gluconokinase but no significant activity of any of these enzymes was found. The revertants, like the wild-type, were also unable to grow on gluconate. Unfortunately, time precluded a more thorough examination of glucose catabolism in these revertants but obviously further study will be of great interest.

It is concluded that, in the wild-type strain, glucokinase is the physiologically significant enzyme used for glucose phosphorylation under the growth conditions, representing a vast excess of glucose used.

This is supported by the fact that some glucose-positive revertants of strain 7009 regained glucokinase activity, and by the fact that all four glucose-negative strains isolated lost glucokinase activity. It is possible that the glk mutations in strains 7013 and 7015 are not revertible by point mutations which would explain the lack of glucokinase-positive revertants amongst the glucose-positive revertants of these strains.

6.2.5 Characterization of strain 7039

In an attempt to identify the carbon source used by the bacteroid, attempts were made to isolate mutants of strain 7013 which were defective in fructose catabolism. Strain 7039 was the only such mutant found from more than five hundred micro-colonies tested after ampicillin enrichment. It was found to differ from strain 7013 only in its inability to grow on fructose (Tables 25 and 26). Strain 7039 contained all the enzymes listed in Table 27 , including fructokinase. It was, however, defective in fructose uptake (genotype fup) (Fig. 8). The uptake of glucose was unaffected (Fig. 6b).

Strain 7039 was able to grow very slowly on fructose (Table 26) and revertants did not arise on plates. Therefore, fructose-positive revertants were isolated by serial culture in RDM containing fructose. This was done by inoculating 20 McCartney bottles, each containing 10 ml of the medium, with separate single colonies, and incubating the cultures until they became turbid. This took between six and ten days. Aliquots (0.1 ml) of these cultures were then subcultured into fresh broths which were once again incubated until the cultures became turbid. A loopful of each of these cultures was then streaked on to RDM-fructose plates and a fast-growing isolate from each plate was purified and tested for fructose uptake. Each of the twenty revertants was able to take up fructose when induced, and seven of the twenty were also able to take up fructose after growth in the absence of fructose, i.e. were constitutive for fructose uptake. This suggests that the original mutation was in a regulatory rather than a structural gene. The slow uptake of fructose by strain 7039 (Fig. 8) may therefore

Fig. 8 Uptake of ^{14}C fructose by strains 7013 and 7039

Bacteria were grown in RDM containing histidine and the specified carbon source, and were assayed for fructose uptake as described in Section 2.11.

○, Strain 7013 grown with 0.4% (w/v) fructose plus 0.4% (w/v) inositol as carbon source.

●, Strain 7039 grown with 0.4% (w/v) fructose plus 0.4% (w/v) inositol as carbon source.

□, Strain 7013 grown with 0.4% (w/v) inositol as carbon source.

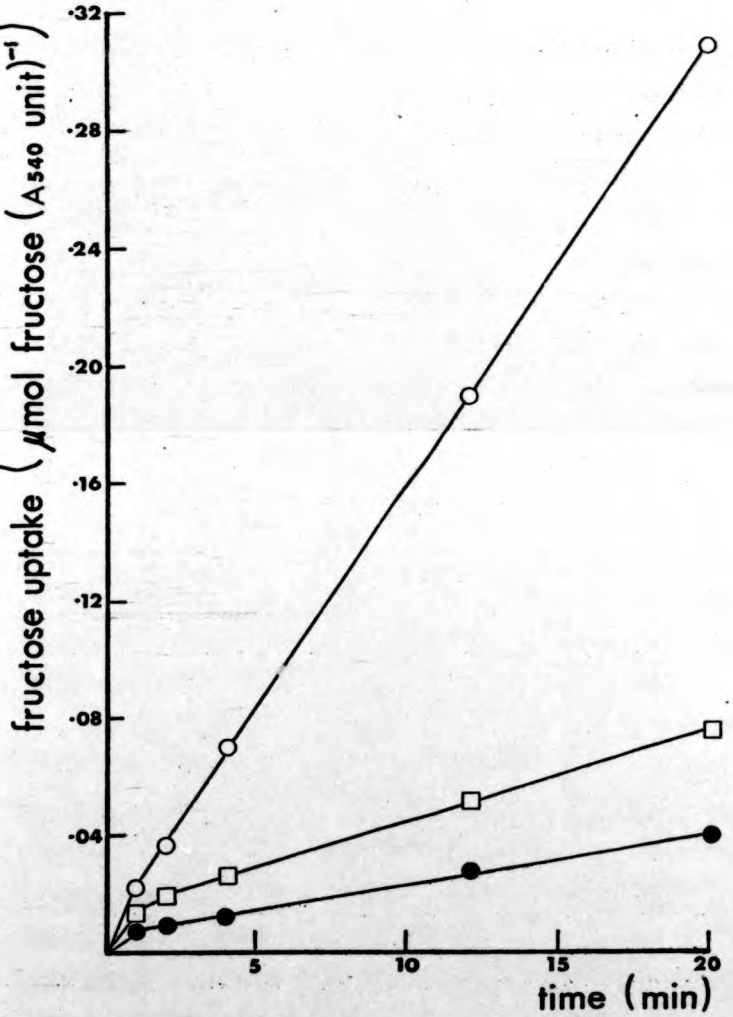
Fig. 8 Uptake of ^{14}C fructose by strains 7013 and 7039

Bacteria were grown in RDM containing histidine and the specified carbon source, and were assayed for fructose uptake as described in Section 2.11.

O, Strain 7013 grown with 0.4% (w/v) fructose plus 0.4% (w/v) inositol as carbon source.

●, Strain 7039 grown with 0.4% (w/v) fructose plus 0.4% (w/v) inositol as carbon source.

□, Strain 7013 grown with 0.4% (w/v) inositol as carbon source.


fructose uptake (μmol fructose (A_{540} unit)$^{-1}$)
·32
·28
·24
·20
·16
·12
·08
·04
5
10
15
20
time (min)

be accounted for by the presence of non-induced amounts of the fructose-permease system.

6.2.6 Characterization of strain 7028

Strain 7028 which was originally isolated on the basis of its fructose-negative phenotype was found also to be impaired in growth on sucrose, glucose, mannose, mannitol and sorbitol (Tables 25 and 26). The growth rate of strain 7028 on solid media containing lactose, galactose, arabinose, ribose, glycerol, arabitol, ribitol or inositol as carbon source was similar to that of the parent strain, 7012. Growth on glucose plus galactose was also normal. Uptake of glucose, fructose and mannitol by induced cultures was also at wild-type initial rates. Revertants (frequency 10^{-7}-10^{-8}) isolated on glucose, fructose or sorbitol (four from each carbon source) regained the ability to grow on all of the carbon sources.

Strain 7028 contained all the enzymes listed in Table 20 except 'ED enzyme' (Fig. 9). In particular, it contained wild-type amounts of G6P dehydrogenase and 6PG dehydrogenase, and its ability to grow normally on ribose and other pentoses and pentitols (see above) indicated that the strain possessed an intact PP pathway.

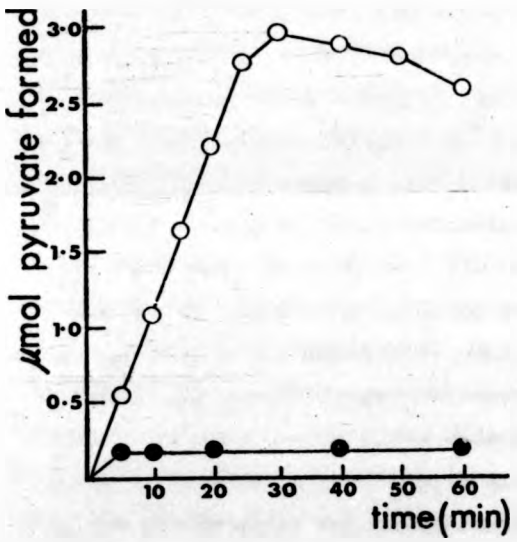
The normal growth of strain 7028 on galactose plus the discovery of galactose dehydrogenase activity suggested that R. trifolii metabolized galactose by a separate pathway, perhaps analogous to that found in Pseudomonas saccharophila (Deley and Doudoroff, 1957). An attempt to find a pathway analogous to the ED pathway by assaying for pyruvate production from galactose in the presence of ATP, $NADP^+$ and $MgCl_2$ in extracts prepared from galactose-grown bacteria of strain 7028 was unsuccessful.

6.2.7 Characterization of strains 7049 and 7056

Strains 7049 and 7056 were unable to grow on any hexose, pentose or triose tested (Table 25). Strain 7056 was also unable to grow on succinate as sole C source, but strain 7049 and the other mutants isolated by the same procedure (Section 5.2.3) retained the ability to

Fig. 9 Activity of 'ED enzyme' in extracts of strains 7000 and 7028

Bacteria were grown in RDM containing 100 μg ml^{-1} histidine, 0.4% (w/v) glucose and 0.4% (w/v) galactose. Extracts were prepared and assayed for 'ED enzyme' as described in Sections 2.6 and 2.9.3.13.
○, strain 7000; ●, strain 7028.


µmol pyruvate formed
3·0
2·5
2·0
1·5
1·0
0·5
10
20
30
40
50
60
time (min)

grow on succinate. Both strains 7049 and 7056 were able to grow on RDM-glucose if the medium was supplemented with 50 $\mu g\ ml^{-1}$ of succinate or malate. The growth of both wild-type and mutant strains was inhibited by 2-oxoglutarate and citrate was toxic. The effect of the supplementation of RDM-glucose with amino-acids on on the growth of strains 7049 and 7056 has been described (Section 5.2.4).

Single-step revertants of strain 7049 isolated on RDM-glucose (frequency 10^{-8}) regained the wild-type growth patterns. However, similar revertants of strain 7056 (frequency $< 10^{-8}$) were unable to grow on succinate, and revertants of strain 7056 isolated on succinate (frequency approximately 10^{-6}) regained the ability to grow on succinate but were unable to grow on RDM-glucose. This suggested that strain 7056 carried at least two mutations, one of which caused the inability to grow on succinate. This second mutation was presumably not at the level of succinate uptake because strain 7056 grew on RDM-glucose supplemented with succinate. The cause of the inability of strain 7056 to grow on succinate was not investigated further in this study, but the ability of the strain to grow on RDM-glucose supplemented with succinate but not on succinate alone suggests that the strain may lack an enzyme required for gluconeogenesis.

The growth properties of strains 7049 and 7056 strongly suggested that the strains lacked an anaplerotic enzyme. Therefore, cell-free extracts, prepared from strain 7000 grown in RDM-glucose, were examined for anaplerotic enzymes. No activity of enzymes of the glyoxalate cycle (isocitrate lyase and malate synthetase) was found in the extracts. The extracts did contain a low amount of malic enzyme activity (approximately 5 $nmol\ min^{-1}(mg\ protein)^{-1}$), but similar extracts of strains 7049 and 7056, prepared from bacteria grown in RDM-glucose supplemented with succinate, also contained this activity. No activity of PEP or pyruvate carboxylase was found using

assays based on spectrophotometric procedures (Canovas and Kornberg, 1969; Young et al., 1969). These assays were complicated by the presence of NADH oxidase in the soluble fraction of the cell-free extracts but the removal of NADH oxidase by heat treatment (Young et al., 1969) or ammonium sulphate precipitation (Canovas and Kornberg, 1969) failed to reveal any carboxylase activity.

Results obtained using an assay procedure (Section 2.9.4.1) based on the incorporation of ^{14}C-labelled bicarbonate into acid-stable material are shown in Table 28 . Strain 7000 showed pyruvate carboxylase activity which was stimulated approximately four-fold by acetyl-CoA, a property shared by many pyruvate carboxylases (Utter and Scrutton, 1969). The activity was abolished by treatment of the extract with avidin, an inhibitor specific for biotin-containing enzymes such as pyruvate carboxylase (Utter and Scrutton, 1969). Strain 7049 lacked pyruvate carboxylase activity (Table 28) and a revertant of strain 7049 regained pyruvate carboxylase activity identical to strain 7000. Results obtained with strain 7056 were identical to those obtained with strain 7019 and are not shown.

Apparent phosphenolpyruvate carboxylase and carboxykinase activities were also found, but these were stimulated by acetyl-CoA and were susceptible to avidin (Table 28). They were therefore dependent on pyruvate carboxylase whose substrate (pyruvate) is generated from phosphoenolpyruvate by the action of pyruvate kinase (EC 2.7.1.40). Furthermore, in strain 7049 both activities were lost, and in revertants both activities were restored along with pyruvate carboxylase.

6.2.8 Symbiotic properties of mutant strains

Strain 7014 was used in place of strain 7009 for nodulation tests because strain 7008 forms a defective symbiosis as a consequence of its requirement for tryptophan (Section 3.2.4). No significant difference in time of onset of nodulation, number of nodules formed,

Table 28 Incorporation of $H^{14}CO_3^-$ by extracts of strain 7000 and strain 7049

Bacteria were grown in RDM containing 0.4% (w/v) glucose and 1 mM succinate. Extracts were prepared and assays done as described in Sections 2.6 and 2.9.4.1. Results are expressed as dpm min^{-1} (mg protein)$^{-1}$.

Reaction mixture	Pyruvate carboxylase		PEP carboxylase***		PEP carboxykinase***	
Strain no.	7000	7049	7000	7049	7000	7049
-pyruvate	226	156	-*	-	-	-
-phosphoenolpyruvate	-	-	96	140	353	215
-ATP	547	142	-	-	-	-
-S-acetyl CoA	1195	129	287	319	1002	284
complete	4897	113	1422	305	2576	251
complete + ATP	-	-	1542	211	2577	125
complete; avidin treated	126	ND**	114	ND	117	ND

* -, not applicable

** ND, not done

*** These activities were probably due to the concerted action of pyruvate kinase and pyruvate carboxylase (see text).

colour of nodules, plant response or in amount of acetylene reduction was noted for any of the strains tested except 7056. Acetylene reduction levels estimated 30 days after inoculation are given in Table 29 . The values are lower than may have been expected for a fully effective symbiosis, which is probably due to the short light period ($8\frac{1}{2}$ h) under which the plants were grown. Earlier experiments in which a few plants inoculated with strains 7012, 7013 and 7028 were grown in the plant-growth room under a 16 h light period gave acetylene reduction values in the range of 25-35 nmol h^{-1} $root^{-1}$. The lower acetylene reduction values given in Table 29 were reflected by relatively poor shoot development of the plants. However, the data in Table 29 are presented because the aim of the experiment was to compare the effectiveness of the mutant strains with the fully effective parent strains 7000 and 7012. In fact, the conditions used, resulting in a limited supply of photosynthate, should have accentuated any reduction in the effectiveness of the mutant strains compared to the wild-type , that resulted from reduced efficiency of the coupling of photosynthate utilization to nitrogen fixation in the nodules formed by the mutant strains.

Strain 7056 formed an ineffective symbiosis. The time of onset of nodulation by strain 7056 was similar to that of strain 7000, but the nodules formed were white and no difference in plant response between plants inoculated with strain 7056 and uninoculated control plants could be detected. The addition of succinate to the seedling medium at the time of inoculation did not allow effective nodulation by strain 7056, but caused the medium to become turbid, presumably due to the growth of contaminant bacteria. The amount of acetylene reduced by nodulated roots of plants inoculated with strain 7056 ranged from 0-10% of that reduced by nodulated roots of plants inoculated with strain 7000. Plants inoculated with strain 7056 were also grown under a 16 h day-length, but the maximum acetylene reduction value obtained was only 2.1 nmol h^{-1} $root^{-1}$. However, the ineffectiveness

Table 29 Acetylene reduction by red clover inoculated with mutant strains

Nodulation tests were done as described in Materials and Methods (Section 2.13). Thirty days after inoculation, plants were removed from the growth room, 2 h into the light period, and entire roots were assayed for acetylene reduction. Each value is the mean ± standard deviation of 5 sample roots, except for strain 7056. The value presented for strain 7056 represents the range found with 20 sample roots.

Strain	Ethylene produced (nmol h^{-1} $root^{-1}$)
7012	9.61 ± 2.54
7013	8.21 ± 2.79
7014	9.92 ± 2.36
7028	7.13 ± 3.10
7039	6.82 ± 1.89
7049	7.75 ± 2.29
7056	0 - 0.95

of the symbiosis formed by strain 7056 was not caused by its lack of pyruvate carboxylase because pyruvate carboxylase-positive revertants of strain 7056 were still ineffective. Similarly, succinate-positive revertants of strain 7056 were also ineffective. It was therefore concluded that strain 7056 had suffered at least three mutations, one causing the loss of pyruvate carboxylase, another the inability to grow on succinate, and the third the ineffectiveness of the strain.

Six nodules (one nodule per test plant) formed by each mutant strain were surface-sterilized, crushed and streaked out on to various media to determine their phenotype. In each case, rhizobia recovered from the nodule had the same phenotype as the strain used for inoculation.

6.3 Discussion

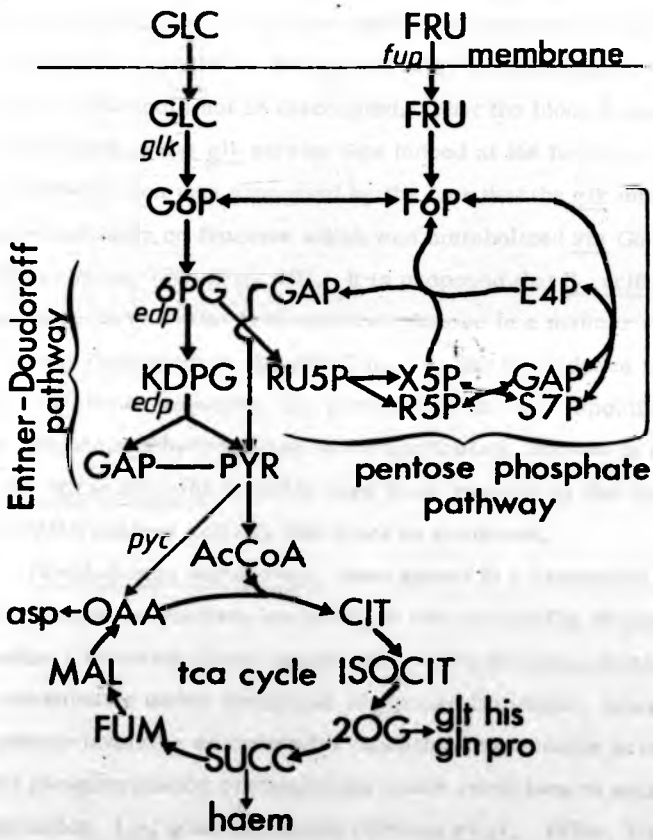
R. trifolii strain 7000 contained key enzyme activities of the Entner-Doudoroff and pentose-phosphate pathways. No evidence for the presence of phosphofructokinase was found, and hence the presence of a complete Embden-Meyerhof-Parnas pathway in R. trifolii could not be demonstrated. This is contrary to a previous report (Katznelson and Zagallo, 1957) but in agreement with Martinez-de Drets and Arias (1972). The latter authors, like myself, were unable to demonstrate significant levels of fructose diphosphate aldolase in most of their strains, but they did not assay for phosphofructokinase. Pathways available to R. trifolii 7000 for carbohydrate catabolism are illustrated in Fig. 10. The sites at which the mutants are blocked are also indicated.

The properties of the glucokinase-negative (glk) mutant strains suggested that the wild-type strain was wholly dependent upon glucokinase for growth on glucose. Since the glk mutant strains were able to take up glucose at similar initial rates to wild-type

Fig. 10

Pathways of glucose and fructose catabolism available to R. trifolii strain 7000. The mutants are blocked at the steps indicated: glk, strains 7009, 7013 and 7039; fup, strain 7039; pyc, strain 7049. Strain 7028 is blocked at one or other of the steps labelled edp.

Abbreviations: GLC, glucose; FRU, fructose; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; 6PG, 6-phosphogluconate; KDPG, 2-keto-3-deoxy-6-phosphogluconate; GAP, glyceraldehyde 3-phosphate; RU5r, ribulose 5-phosphate; X5P, xylulose 5-phosphate; R5P, ribose 5-phosphate; S7P, sedoheptulose 7-phosphate; E4P, erythrose 4-phosphate; PYR, pyruvate; AcCoA, acetyl-CoA; OAA, oxaloacetate; CIT, citrate; ISOCIT, isocitrate; 2OG, 2-oxoglutarate; SUCC, succinate, FUM, fumarate; MAL, malate; tca, tricarboxylic acid; glt, glutamate; gln, glutamine; his, histidine; pro, proline; asp, aspartate.


GLC
FRU
fup
membrane
GLC
glk
FRU
G6P
F6P
6PG
GAP
E4P
edp
Entner-Doudoroff pathway
KDPG
RU5P
X5P
GAP
R5P
S7P
edp
GAP
PYR
pentose phosphate pathway
pyc
AcCoA
asp
OAA
CIT
MAL
tca cycle
ISOCIT
FUM
2OG
glt his gln pro
SUCC
haem

(Fig. 6), R. trifolii, in common with other obligately-aerobic bacteria (Romano et al., 1970) did not appear to contain a PEP: glucose phosphotransferase system (PTS). However, the finding that many glucose-positive revertants of the glk strains did not regain glucokinase activity suggested that R. trifolii possessed the potential for an alternative pathway of glucose metabolism, although the possibility that the revertants synthesized unstable glucokinase could not be discounted. That the block in glucose metabolism in the glk strains was indeed at the level of phosphorylation was supported by the fact that the glk mutants grew normally on fructose which was metabolized via G6P and 6PG in strain 7000 (Fig. 10). It is proposed that R. trifolii may possess the potential to metabolize glucose in a manner similar to many Pseudomonas species (Fig. 7), but no evidence for this was obtained. However, the presence of an NAD^+ specific glucose or gluconate dehydrogenase in the particulate fraction of the cell-free extracts would probably have been masked by the large amount of NADH oxidase activity this fraction contained.

Pseudomonas aeruginosa, when grown in a chemostat, regulates its glucose metabolism according to the availability of glucose. The pathway involving direct uptake followed by phosphorylation of glucose predominates under conditions of glucose limitation, whereas the pathway involving extracellular oxidation to gluconate prior to uptake and phosphorylation predominates under conditions of ammonia limitation, i.e. glucose excess (Whiting et al., 1976a, b). It is possible that R. trifolii 7000 metabolizes glucose only by the former pathway when grown on plates or in batch culture, either because it has lost the potential for the latter pathway through mutation or because the latter pathway is tightly repressed under the conditions used. However, the inability of the glucose-positive, glucokinase-negative revertants to grow on gluconate suggests that a pathway involving extracellular oxidation of glucose to gluconate

does not exist in the revertants. However, a pathway involving intracellular oxidation of glucose to gluconate cannot be discounted, but if such a pathway exists in the revertants, R. trifolii strain 7000 would have to be defective in gluconate uptake. The ability of Rhizobium strains in general to grow on gluconate does not seem to have been studied, but R. japonicum possesses both an intracellular gluconokinase and gluconate dehydrogenase when grown on gluconate (Keele et al., 1970). Obviously, further study of the initial steps of glucose metabolism in R. trifolii and especially in the glucose-positive, glucokinase-negative revertants is required.

The glk mutant strains of R. trifolii showed a pleiotropic growth phenotype (see Fig. 5). Studies on this pleiotropy are reported in Section 7.2.1. However, it is worth noting here that the strains were unable to grow on sucrose, the major sugar transported to the nodules.

Strain 7028, deficient in either 6-phosphogluconate dehydratase or KDPG aldolase, lost the ability to grow on glucose and was severely impaired in its ability to grow on other six-carbon sugars except galactose. This indicates that the ED pathway plays a major role in the metabolism of six-carbon sugars in R. trifolii 7000 and provides further evidence for the absence of a functional EMP pathway. The mutant grew very slowly on sucrose and on the six-carbon sugars except glucose, possibly via the PP pathway, the key enzyme of which, $NADP^+$-dependent 6PG dehydrogenase, was present at high specific activity. Indeed, the level of $NADP^+$-6PG dehydrogenase was such that no impairment of growth might be expected. However, a functional ED pathway may be necessary for growth for a number of reasons. In Escherichia coli accumulated KDPG formed in KDPG aldolase mutants is inhibitory, thereby inhibiting growth on gluconate despite the availability of the PP pathway (Fradkin and Fraenkel, 1971). However, the normal growth of strain 7028 on glucose plus galactose and on lactose, the catabolism of which generates glucose internally, indicates that an inhibitory compound was not accumulated in this strain.

I am unaware of any report of an organism catabolizing six-carbon sugars solely *via* the PP pathway under normal conditions, and it may be that sole use of the pathway causes metabolic imbalance, *e.g.* that it is too inefficient to support growth with only one mole of acetyl-CoA produced per mole of glucose. Another possibility is that sole use of the PP pathway may lead to an excess of NADPH being generated, resulting in a shortage of $NADP^+$ which is required as co-factor for G6P dehydrogenase, 6PG dehydrogenase, glyceraldehyde - 3-phosphate (GAP) dehydrogenase and isocitrate dehydrogenase in *R. trifolii* (Table 20). A similar explanation has been put forward for the inability of glucosephosphate isomerase mutants of *E. coli* to grow on glucose despite the availability of the PP pathway (Fraenkel and Vinopal, 1973). Nothing is known about the re-oxidation of NADPH or the control of glucose catabolism in *Rhizobium*. It is therefore possible that the ratio of NADPH to $NADP^+$ controls the participation of the PP pathway in hexose catabolism in wild-type *R. trifolii*, resulting in the inhibition of the pathway in strain 7028. The normal growth of strain 7028 on lactose may be explained by the complete oxidation of the galactose moiety of lactose, which may relieve the metabolic imbalance. Nevertheless, strain 7028 did not grow equally well on all carbon sources whose catabolism should be equally impaired (Table 26) and investigation of this point is required.

A further possibility would be a pathway of fructose catabolism such as is found in several species of *Pseudomonas* (Sawyer *et al.*, 1977). These species catabolize fructose *via* a PEP-dependent conversion to fructose 1-phosphate followed by ATP-dependent formation of fructose 1,6-diphosphate by 1-phosphofructokinase. The fructose diphosphate is then catabolized primarily by the ED pathway but to a lesser extent by the EMP pathway since 6-phospho-fructokinase is not required (Sawyer *et al.*, 1977). This pathway of fructose catabolism is largely used to catabolize exogenously-

supplied fructose, but is also induced to a lower level in bacteria grown on mannitol, which generates fructose endogenously (Phibbs et al., 1978). Catabolism of mannitol also induces fructokinase in P. aeruginosa (Phibbs et al., 1978). Since the catabolism of sucrose, fructose and sorbitol in Rhizobium generates fructose (Martinez-de Drets and Arias, 1970; Martinez-de Drets et al., 1974), whereas the catabolism of glucose presumably does not, the occurrence of a fructose 1-phosphate pathway could explain the slow growth of strain 7028 on sucrose, fructose and sorbitol, i.e. the catabolism of these sugars in strain 7028 would occur via a modified EMP pathway rather than the PP pathway. These possibilities could be distinguished by radiorespirometric studies. Nevertheless, the occurrence of 6PG dehydrogenase in strain 7028 suggests that carbohydrate catabolism in this strain occurs via the PP pathway and thus carbohydrate metabolism in R. trifolii may be similar to that occurring in the closely-related species Agrobacterium tumefaciens. In this species approximately 45% of the glucose is catabolized via the PP pathway and the remainder is catabolized via the ED pathway (Arthur et al., 1973).

Mutants in the ED pathway have been described in other genera lacking the EMP pathway. Blevins et al. (1975) isolated mutants of P. aeruginosa deficient in 6PG dehydratase which were unable to grow on glucose; however, these mutants also lost an apparent $NADP^+$-linked 6PG dehydrogenase activity which these workers ascribed to GAP dehydrogenase activity, the GAP being produced from 6PG via the ED pathway. No functional PP pathway was found in these strains. Vicente and Canovas (1973) isolated similar mutants in P. putida, but although stating that the PP pathway was present, did not present data for 6PG dehydrogenase.

The normal growth of strain 7028 on lactose and on galactose suggested that R. trifolii catabolizes galactose by a different pathway. We have identified both $NADP^+$ and NAD^+-galactose dehydrogenase

activities which may initiate a pathway similar to that initiated by the NAD^+-linked galactose dehydrogenase (EC1.1.1.48) in Pseudomonas saccharophila (DeLey and Doudoroff, 1957). This pathway is analogous to the ED pathway of glucose catabolism. Escherichia coli utilizes galactonate by a similar pathway (Deacon and Cooper, 1977; Cooper, 1978) and E. coli mutants containing an NAD^+-linked galactose dehydrogenase have been found (Wu, 1976). A dehydrogenase with dual co-enzyme specificity which acted on L-arabinose, fucose and galactose was reported to be part of the L-arabinose catabolic pathway in R. japonicum (Pedrosa and Zancan, 1974), but the pathway of galactose catabolism has not yet been studied in Rhizobium.

The effective symbiotic properties of strains 7013, 7014 and 7015 suggest that glucose is not catabolized by the bacteroids to provide the necessary ATP and reductant for nitrogen fixation. Strain 7039 was isolated from strain 7013 and was defective in the uptake of fructose, the other product of invertase. The effectiveness of this strain strongly suggests that neither sucrose, glucose nor fructose are used by the bacteroids, although the possibility that fructose and/or glucose are taken up by different mechanisms, perhaps involving phosphorylation, by the bacteroid could not be discounted. However, the effective symbiotic properties of strain 7028 provide further evidence that glucose or fructose catabolism by the bacteroid is not necessary for an effective symbiosis to be formed, and also suggest that metabolites of glucose such as glucose 6-phosphate are not provided to the bacteroid by the plant.

The findings that R. trifolii did not possess phosphofructokinase but metabolized glucose primarily by the ED pathway, and that glucose was not metabolized by the bacteroids, are in contrast to results presented for R. japonicum by Mulongoy and Elkan (1977a). These workers reported simultaneous operation of the ED and EMP pathways in R. japonicum and suggested that the efficiency of nitrogen fixation was correlated with the use of the EMP pathway.

However, they assayed phosphofructokinase by coupling the formation of ADP to pyruvate kinase and lactate dehydrogenase, rather than by measuring the formation of fructose diphosphate. Their method of assay may therefore by subject to error, e.g. interference by NADH oxidase; note also the apparent PEP carboxylase activities reported in Table 28 which resulted from pyruvate formation from PEP (see Section 6.2.7). Mulongoy and Elkan (1977a, b) confirmed that R. japonicum did not possess $NADP^+$-dependent 6PG dehydrogenase but also reported that their strains did possess an NAD^+-dependent 6PG dehydrogenase, the product of which was a phosphorylated six-carbon compound containing ketonic group(s). The enzyme was also found at low activity in fast-growing species including R. trifolii (Mulongoy and Elkan, 1977b), and it was suggested that the enzyme might initiate a new catabolic pathway (Mulongoy and Elkan, 1977a). Although NAD^+-dependent 6PG dehydrogenase was not assayed in this study, the growth properties of strain 7028 suggest that if it did occur, it did not play a significant role in glucose catabolism. It apparently also did not play a significant role in R. japonicum (Mulongoy and Elkan, 1977a).

The properties of strain 7049 show that pyruvate carboxylase is the physiologically important anaplerotic enzyme used by R. trifolii for growth on carbon sources metabolized through to acetyl-CoA. The enzyme plays a similar role in the closely related Agrobacterium tumefaciens (Chern et al., 1976) and in P. aeruginosa (Phibbs et al., 1974). The anaplerotic enzyme is required to replace tricarboxylic acid cycle intermediates used in amino-acid and haem biosynthesis (Kornberg, 1966). The symbiotic properties of histidine-negative (Section 3.2.4) and glutamate-negative mutants (Kondorosi et al., 1977) show that the nodule cytosol provides adequate levels of amino-acids normally

synthesized from the intermediates. However, bacteroids are very active in synthesizing the haem portion of leghaemoglobin (Cutting and Schulman, 1969; Godfrey et al., 1975; Nadler and Avissar, 1977). Hence the effective symbiotic properties of the pyruvate carboxylase-negative mutants suggest that the bacteroids receive a supply of tricarboxylic acid intermediates from the nodule cytosol. This, coupled with the symbiotic properties of the other hexose-negative mutants, suggests that tricarboxylic acid cycle intermediates may be the major substrate received by the bacteroid and used to provide the ATP and reductant required for nitrogen fixation. This suggestion is supported by the observation that succinate was the most effective substrate for enhancing nitrogen fixation by bacteroids in vitro (Bergersen and Turner, 1967).

Free-living R. trifolii accumulates large amounts of the storage polymer poly-β-hydroxybutyrate (PHB) (Vincent, 1977) but there is little evidence for the nature or role of storage polymers in clover bacteroids. Clover bacteroids are largely devoid of PHB although older bacteroids may contain a few granules (Dart, 1977) and ineffective bacteroids have been shown to accumulate glycogen which was not found in effective bacteroids (Bergersen, 1955). Nevertheless, catabolism of both of these polymers leads to the production of acetyl-CoA, which to be completely oxidized requires a functional tricarboxylic acid cycle. An impaired symbiosis by strain 7049 might be predicted if a storage polymer is used to provide energy for nitrogen fixation during dark periods. Thus the effective symbiotic properties of strain 7049 complement the findings of Wong and Evans (1971) who suggested, after studying PHB depletion in soy bean nodules during prolonged periods of darkness, that the storage polymer was not used by the bacteroids to support nitrogen fixation. However, Kretovich et al. (1977) found an inverse relationship between the PHB content and the nitrogen-fixing activity of bacteroids formed by R. lupini and R. leguminosarum and suggested that the PHB was utilized during nitrogen fixation.

Nevertheless, the significance of their data is difficult to assess. The authors suggested that the PHB was used to provide the carbon skeleton required to bind ammonia, but in view of the evidence that the assimilation of ammonia occurs in the nodule cytosol (see Section 5.5), this seems unlikely. Obviously research into the factors leading to an accumulation of PHB in the energy-limited bacteroids is required, since the polymer is normally synthesized as an energy reserve when carbon substrates are in excess. However, it may also be synthesized in response to oxygen limitation (see review by Dawes and Senior, 1973). Studies on polymer accumulation by bacteroids of strain 7049 could also be of interest.

6.4 Carbohydrate metabolism in rhizobia: current knowledge and conclusions

No studies on central carbohydrate metabolism in rhizobia have been reported since this study was begun, apart from those of Mulongoy and Elkan, (1977a, b). Thus, current knowledge suggests that central carbohydrate metabolism in both slow-growing (Keele et al., 1969, 1970; Mulongoy and Elkan, 1977a) and fast-growing rhizobia (this study) occurs primarily by the ED pathway. Contrary to some reports (Katznelson and Zagello, 1957; Mulongoy and Elkan, 1977a; reviews by Jordan (1962) and Vincent (1977)), there is no evidence for the operation of the EMP pathway in rhizobia (Keele et al., 1969, 1970; Martinez-de Drets and Arias, 1972; this study). The oxidative PP pathway which is not present in slow-growing rhizobia (Keele et al., 1969, 1970; Martinez-de Drets and Arias, 1972; Mulongoy and Elkan, 1977a, b) may play a minor role in carbohydrate metabolism in fast-growing rhizobia (Martinez-de Drets and Arias, 1972; this study). Radiorespirometric studies of hexose metabolism in fast-growing rhizobia are obviously required, and the inclusion of strain 7028 in such studies would be of interest. If, as anticipated, hexose metabolism in this strain is by the PP pathway, this would constitute positive proof for the absence of a

catabolically-active EMP pathway in R. trifolii. However, the total lack of growth of strain 7028 on glucose will complicate such studies. Nevertheless, studies using [1-^{14}C]-labelled fructose should distinguish between an EMP pathway (possibly involving fructose 1-phosphate) and the PP pathway.

Carbohydrate metabolism in R. trifolii 7000 resembles that of various Pseudomonas species in several respects. For example, P. aeruginosa catabolizes glucose via the ED pathway (Blevins et al., 1975) and requires pyruvate carboxylase for growth on hexoses (Phibbs et al., 1974). It also phosphorylates glucose via glucokinase at least under some conditions (e.g. Whiting et al., 1976a, b), and also oxidizes mannitol prior to phosphorylation (e.g. Phibbs et al., 1978). Galactose metabolism may also be similar in the two genera (this study). All these aspects of carbohydrate metabolism differ from those found in enteric bacteria (reviewed by Fraenkel and Vinopal, 1973) and suggest that carbohydrate metabolism in Pseudomonas will provide a better model for future studies of carbohydrate metabolism in Rhizobium.

6.5 Carbohydrate metabolism in nodules: current knowledge and speculation

The effective symbiotic properties of the various mutant strains do not support the proposition of Bergersen (1974, 1977b) that bacteroids receive hexoses as their major energy substrate. It is more likely that bacteroids receive a tricarboxylic acid cycle intermediate(s) (this study; Bergersen and Turner, 1967), but final proof of this has not yet been obtained. Certainly no studies relevant to this problem have been reported since this study was begun, which is surprising considering the importance of carbohydrate metabolism in overall nodule function.

A synthesis of the major metabolic processes occurring within the mature nodule cell is presented in Fig. 11. The reactions involved in the assimilation of ammonia are presented in detail in

Fig. 11 Diagrammatic representation of major metabolic processes occurring in the mature nodule cell

Key: A, bacteroid membrane
B, peribacteroid space
C, peribacteroid membrane
D, plant cell membrane

1: invertase
2: enzymes of the Embden-Meyerhof-Parnas pathway
3: PEP carboxylase
4: pyruvate kinase plus pyruvate dehydrogenase
5: citrate synthase
6: enzymes of the tricarboxylic acid cycle
7: electron carriers specific to nitrogenase
8: electron carriers involved in oxidative phosphorylation
9: nitrogenase
10: enzymes involved in ammonia assimilation (see Fig. 4).

The abbreviations not given in the legend to Fig. 10 are: Apo. Lb; apoleghaemoglobin; Lb, leghaemoglobin; PEP, phosphoenolpyruvate.

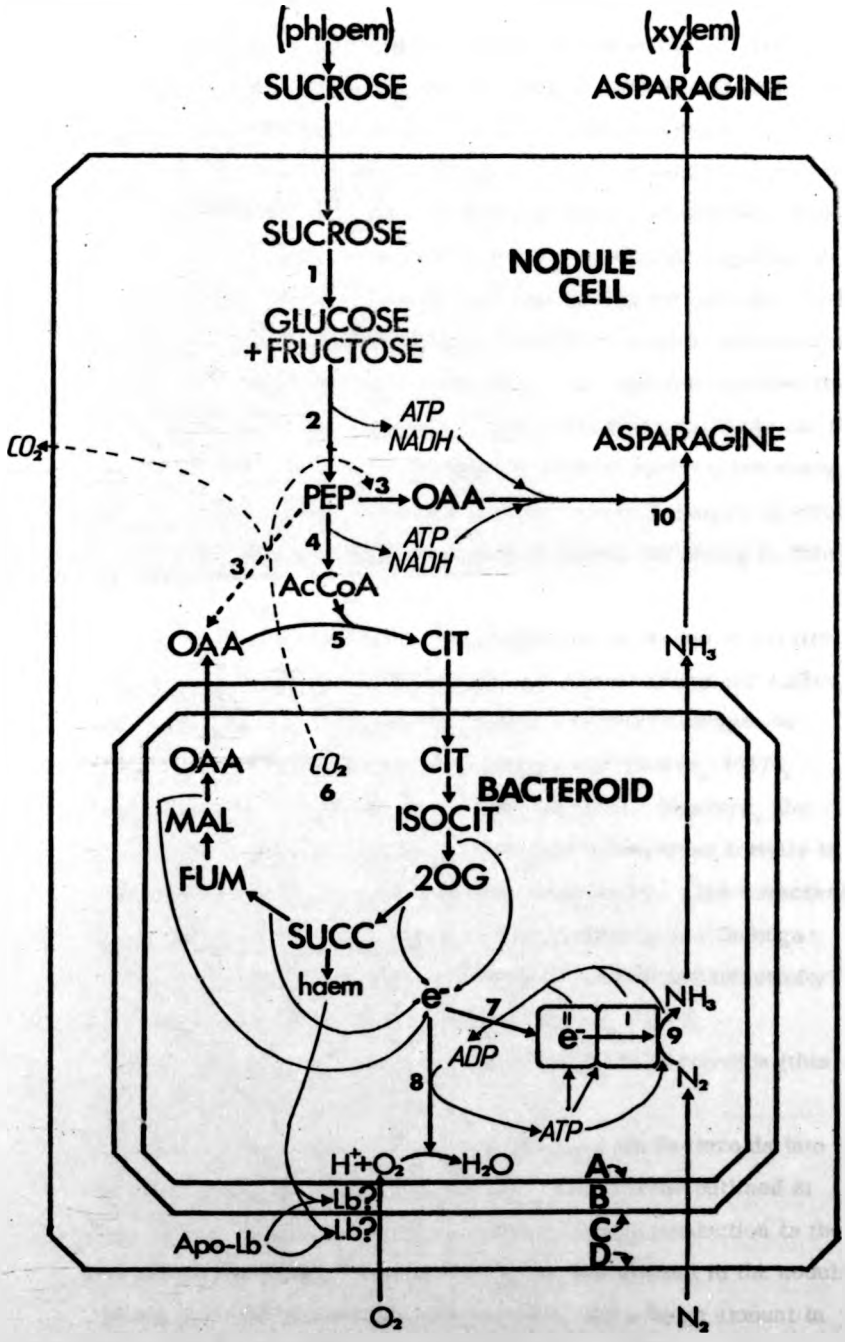

(phloem)
SUCROSE
(xylem)
ASPARAGINE
SUCROSE
1
NODULE CELL
GLUCOSE + FRUCTOSE
2
ATP
NADH
ASPARAGINE
CO2
PEP
3
OAA
10
4
ATP
NADH
3
AcCoA
OAA
5
CIT
NH3
OAA
CO2
6
CIT
BACTEROID
MAL
ISOCIT
FUM
2OG
SUCC
haem
e-
7
NH3
e-
9
ADP
8
N2
ATP
H+ + O2
H2O
A
B
Lb?
Lb?
C
Apo-Lb
D
O2
N2

Fig. 4. (Section 5.5). Some of the processes in Fig. 11 are speculative but most are supported by experimental evidence. The effective symbiotic properties of the mutant strains described in the preceding sections suggest that sequences 1 to 5 occur in the nodule cytosol. In terms of energy production, this is also logical because the EMP pathway is generally regarded as a more efficient producer of energy than the ED pathway (reviewed by Doelle, 1969)). Although the actual tricarboxylic acid cycle intermediate(s) received by the bacteroids is unknown, it is logical to suppose that it is either citrate or isocitrate. These are the most likely for the following reasons:- (i) the bacteroids require vastly more energy than the nodule cytosol as they are the site of nitrogenase function, therefore the more energy-producing reactions occurring in them the better;

(ii) the amount of isocitrate dehydrogenase increases in parallel with the amount of nitrogenase in the bacteroids (Kurz and LaRue, 1977). However succinate and fumarate support nitrogenase activity in isolated bacteroids (Bergersen and Turner, 1967), indicating that the bacteroids can take these up. However, the capacity of citrate or isocitrate to support nitrogenase activity in bacteroids has not, to my knowledge, been tested. The occurrence of reaction 5 in the nodule cytosol is supported by two findings:

(i) pyruvate only supported very low levels of nitrogenase activity in isolated bacteroids (Bergersen and Turner, 1967);

(ii) an anaplerotic enzyme is not required in the bacteroids (this study; also Johnson et al., 1966).

Therefore, the excretion of oxaloacetate from the bacteroids into the nodule cytosol would seem logical. The scheme outlined in Fig. 11 would allow the plant to partition energy production to the site where the energy is required, i.e. a low amount in the nodule cytosol for use in ammonia assimilation, and a large amount in the bacteroids for use in nitrogen fixation.

The proposed scheme is amenable to experimental testing. Of especial interest would be the amounts and regulation of the tricarboxylic acid cycle enzymes in the nodule cytosol, the capacity of bacteroids to take up the various tricarboxylic acid cycle intermediates from very low concentrations, the ability of these intermediates to support nitrogen fixation in the bacteroids and the ability of the bacteroids to excrete oxaloacetate. The isolation of mutants of rhizobia defective in the uptake of the various intermediates should also provide information pertinent to the scheme.

On a wider front, studies of the mechanism by which bacteroids switch off, if in fact they do, their glucose assimilatory enzymes may provide information on the regulation of carbohydrate metabolism in the nodules. These studies could also be done with free-living rhizobia. I was precluded from doing such studies by the inability to obtain growth of the bacteria in broths containing succinate as sole carbon source, but other species of Rhizobium will grow on succinate as sole carbon source (e.g. R. leguminosarum, Beringer (1974); R. japonicum, Tuzimura and Meguro, 1960). In fact, evidence suggesting that succinate-grown bacteria do switch off their glucose assimilatory enzymes was found by Tuzimura and Meguro (1960) who demonstrated that such bacteria oxidize glucose only poorly compared to glucose-grown bacteria.

6.6 Further conclusions

The work described in this chapter has significantly advanced knowledge of carbohydrate metabolism in R. trifolii. The propositions that the EMP pathway plays a significant role in glucose catabolism in the species, and that hexoses are the major energy substrates received by the bacteroids, are no longer tenable. The work also opened up several avenues for future research as described in the preceding three sections (Sections 6.3, 6.4 and 6.5).

CHAPTER 7

STUDIES ON THE REGULATION OF CARBOHYDRATE CATABOLISM IN R. TRIFOLII

7.1 Introduction

The regulation of carbohydrate catabolism in Rhizobium has not been studied; the only hint of regulation was the finding of reduced amounts of mannitol dehydrogenase in R. meliloti when the bacteria were grown in the presence of both mannitol and glucose (Martinez-de Drets and Arias, 1970; reviewed in Section 6.1). The purpose of the studies reported in this chapter was to provide information on the regulation of carbohydrate metabolism in R. trifolii.

The regulation of carbohydrate catabolism has, of course, been extensively studied in other genera. When presented with two carbon sources, many organisms utilize the one that is the better growth substrate preferentially to the other. Biphasic growth curves are often observed under these conditions and result from one sugar being completely metabolized before the metabolism of the second commences. This phenomenon was first studied in E. coli wherein it was found that the inclusion of glucose in the medium reduced the concentration of some catabolic enzymes. This was called the glucose effect. Intensive study of the glucose effect has led to a complexity of data and terminology describing phenomena which may or may not be inter-related or independent. The purpose of this introduction is to define the terminology used and to give an outline of the basic phenomena and the mechanisms which may underlie them. Most of the work on this subject has been done with enteric bacteria and has been the subject of a number of recent reviews (e.g. Magasanik, 1970; Postma and Roseman, 1976; Kornberg and Jones-Mortimer, 1977; Saier, 1977; Saier and Moczydlowski, 1978).

The glucose effect, which is not limited to glucose but is exhibited by other good carbon sources, has been subdivided by Magasanik (1970) into three main effects: catabolite repression, transient repression and inducer exclusion. Catabolite repression refers to

the permanent though often weak inhibition of enzyme synthesis found when induced cells are grown in the presence of a repressing sugar and the inducing sugar. It is thought to be caused by catabolites of the repressing sugar present in the cell, although these catabolites have not been identified. However, there is a correlation such that the better the repressing sugar is as a carbon source the more intense the catabolite repression it exerts, suggesting that the intensity of the carbon flux through the catabolic pathways is important. The level of cyclic AMP (cAMP) in the cell is also important because the addition of cAMP to the medium in which the cells are growing can overcome catabolite repression. An increased amount of the inducing sugar can also overcome catabolite repression (reviews by Magasanik (1970) and Kornberg and Jones-Mortimer (1977)), suggesting that high cAMP levels may balance low inducer levels to allow transcription. The requirement for cAMP in the synthesis of inducible enzymes was shown by Perlman and Pastan (1969) who found that mutants unable to synthesize cAMP due to a deficiency in adenylate cyclase were unable to utilize many carbohydrates for which inducible catabolic pathways were required. Evidence now suggests that the extent of catabolite repression of an enzyme may be inversely reflected by the intracellular steady-state concentration of cAMP (reviewed by Kornberg and Jones-Mortimer, 1977). However, catabolite repression reversible by cAMP has been demonstrated in Bacillus megaterium, an organism which does not contain cAMP (Ullmann, 1974). Phosphorylated intermediates may be involved in catabolite repression in Bacillus (Lopez and Thoms, 1977).

Catabolite repression is therefore expressed when a good carbon source is added to a culture growing on a poor carbon source requiring an induced enzyme for catabolism. However, at the time the good carbon source is added, the synthesis of that induced enzyme is halted for a variable period (0.1-0.5 generations) before the synthesis begins again at the differential rate

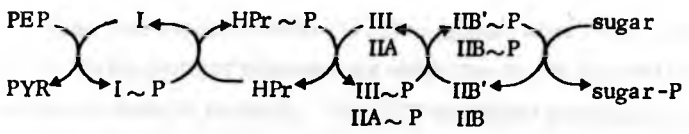
characteristic of catabolite repression. This temporary but strong repression is termed transient repression and, in contrast to catabolite repression, catabolism of the repressing sugar is not required for its manifestation. However, it is reversible by cAMP, but in this case the transport system for the repressing sugar may be involved. For example, mutants lacking the Enzyme II (see later for definition) specific for a sugar are insensitive to transient repression by that sugar (Magasanik, 1970). Peterkofsky (1977) and Gonzalez and Peterkofsky (1977) have proposed that transient repression may be mediated by an interaction of the enzyme I of the PEP:sugar phosphotransferase system (PTS) and adenylate cyclase, where phosphorylated Enzyme I activates adenylate cyclase and dephosphorylated Enzyme I inhibits it. The involvement of the PTS in the regulation of adenylate cyclase is further discussed later.

In experiments demonstrating transient and catabolite repression, glucose is added to a culture already expressing the enzyme in question. If the experiment is carried out differently by adding glucose and inducer simultaneously to a culture not expressing the enzyme in question, induction of the enzyme is not observed. It was shown that glucose could prevent the entry of the inducer into the cell. This phenomenon is called inducer exclusion. The molecular mechanism for inducer exclusion is also unknown but the permeases for both sugars are probably involved (see below).

McGinnis and Paigen (1969, 1973) adopted a different approach to the regulation of sugar utilization by measuring the effect of the addition of glucose on the rate at which E. coli utilized a number of carbon sources. Both the production of $^{14}CO_2$ from the labelled sugar and the incorporation of label into acid-stable material were measured. The effect of glucose on the utilization of seven carbon sources was tested, and in each case glucose caused an immediate and reversible inhibition of the carbon source utilization. This

effect was termed catabolite inhibition and was different from catabolite repression because the activity rather than the synthesis of a cellular constituent was the target of the regulation. Also, only glucose and glucose 6-phosphate caused the effect. Catabolite inhibition was proposed to be a manifestation of transport regulation in that the uptake of the affected sugar was inhibited (McGinnis and Paigen, 1973). Amaral and Kornberg (1975) found a similar effect of glucose on the uptake of fructose by cultures previously exposed only to fructose. Their data are discussed in detail later.

In many organisms the PTS mediates the transport concomitant with phosphorylation of many but not all carbohydrates. The system is comprised of a series of proteins and is thought to function in the sequence outlined below (Postma and Roseman, 1976; but see Kornberg and Jones-Mortimer, 1977, for various caveats).



The proteins Enzyme I and HPr are soluble proteins used for the uptake of all sugars transported by the system whereas the enzyme II and III complexes are sugar-specific. The enzymes IIA, IIB and IIB' are membrane-bound whereas the enzymes III are soluble. Some sugars are taken up by a IIA/IIB type system whereas others are taken up by a III/IIB' type system. Both types of system occur for the uptake of glucose (see reviews by Postma and Roseman, 1976; Kornberg and Jones-Mortimer, 1977).

An involvement of the PTS in the regulation of catabolic enzyme synthesis was shown by the fact that certain pts mutants of E. coli and S. typhimurium (deficient in Enzyme I or HPr) were unable to grow on some sugars which were not substrates of the PTS (non-PTS sugars), and that this lack of growth was due to exclusion of

the non-PTS sugars from the cell. Some pts mutants, which were found to be leaky in that they synthesized low amounts of the affected PTS protein, were able to grow on the non-PTS sugars, but the synthesis of the relevant catabolic enzymes was hypersensitive to repression by PTS sugars (Saier and Roseman, 1976a; Saier et al., 1976a). This repression could in some cases be overcome by the addition of cAMP (Pastan and Perlman, 1969, 1970) and in fact the activity of adenylate cyclase was found to be subject to co-ordinate regulation by the PTS (Saier and Feucht, 1975; Castro et al., 1976; Saier et al., 1976b; Gonzalez and Peterkofsky, 1977; Peterkofsky, 1977; Saier, 1977). However, the involvement of cAMP and adenylate cyclase in the various modes of regulation of catabolic enzyme synthesis (catabolite repression, transient repression, PTS-mediated repression) has still not been completely resolved (see Postma and Roseman, 1976; Kornberg and Jones-Mortimer, 1977).

Saier and Moczydlowski (1978) suggested that there are at least three distinct mechanisms operative in the control of sugar uptake in enteric bacteria. The PTS-mediated regulation, as described above, depends upon the catalytic activities of the protein components of the PTS and operates only on certain non-PTS sugar transport systems. PTS-mediated repression is observed in wild-type cells but sensitivity to repression by PTS sugars (i.e. sugars taken up by the PTS system) is greatest in mutants containing the least amount of Enzyme I (pts I) or HPr (pts H) (Saier et al., 1976a). PTS-mediated repression can be alleviated by the introduction of a second-site mutation into the pts mutant strains. Strains carrying one such mutation (crr) were completely resistant to PTS-mediated repression of catabolic enzyme synthesis. The crr mutants either contained depressed activity of, or were completely deficient in, a particular sugar-specific protein of the PTS involved in the phosphorylation of glucose and α-methylglucoside, and termed

Enzyme III^{glc} (Saier and Roseman, 1976b). A second class of mutants carrying a second-site mutation were resistant to PTS-mediated repression exerted by a specific PTS sugar only, and were deficient in the Enzyme II for that sugar (Saier and Roseman, 1976a; Saier et al., 1976b). The third class of the second site mutations were mapped in or near the gene specifying the permease for the affected non-PTS sugar and the activity of the transport system for that specific non-PTS sugar was insensitive to regulation by PTS sugars (Saier and Roseman, 1976b; Saier et al., 1978). The utilization of the specific non-PTS sugar by these mutants was also insensitive to catabolite inhibition and the synthesis of the corresponding catabolic enzymes was partially resistant to catabolite repression by the PTS sugars (Saier et al., 1978). This latter class of mutants suggested that some permeases were allosteric regulatory proteins which could be indirectly modulated by the PTS and that inhibition of inducer uptake was a primary cause of the repression of catabolic enzyme synthesis, at least in Salmonella typhimurium, but probably also in E. coli (Saier et al., 1978).

These mutant analyses therefore suggested that PTS-mediated regulation of transport activity by a particular sugar depended on: (i) the enzyme II complex specific for that sugar; (ii) the product of the crr gene; and (iii) a permease-specific gene product, which may correspond to a regulatory site of the target transport system. The model shown in Fig. 12 was therefore proposed for the PTS-mediated regulation of transport activity in S. typhimurium (Saier and Moczydlowski, 1978). The essential features of the model are that RPr physically interacts with the proposed allosteric regulatory site of the sensitive transport system. It is assumed that RPr can be phosphorylated by the transfer of a phosphoryl group from phospho-HPr with the resultant formation of a high-energy phosphoprotein. Only the non-phosphorylated form of RPr will bind to the allosteric binding site of the permease system and depress transport activity. This model accounts for most

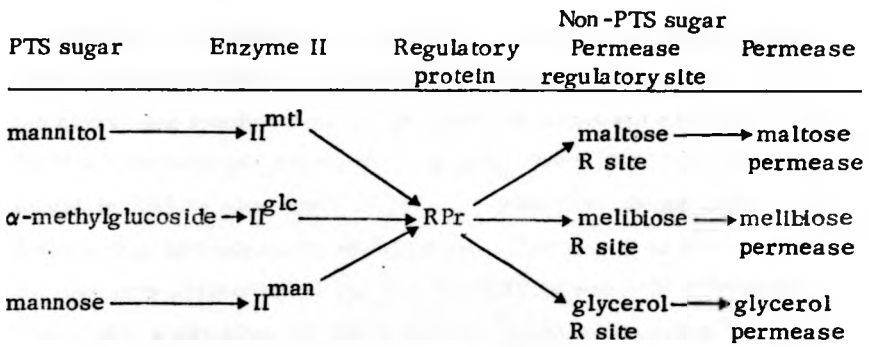


Fig. 12 Schematic representation of a hypothesis for the mechanism of PTS-mediated inhibition of transport activity in S. typhimurium. II represents an enzyme II complex of the PTS and RPr represents the product of the crr gene and may be enzyme III^{glc} (from Saier and Moczydlowski, 1978).

observations. For example, in the absence of a PTS sugar, RPr would be phosphorylated, but the addition of a PTS sugar would drain the phosphate from RPr to the sugar. In leaky pts I or pts H mutants, the rate at which RPr could be phosphorylated should be depressed, resulting in a lower concentration of the PTS sugar being effective in dephosphorylating RPr and therefore inhibiting transport. Tight pts I or pts H mutants would be unable to phosphorylate RPr, resulting in their inability to grow on the non-PTS sugars. In wild-type cells, high concentrations of PTS-sugars would deplete phospho-RPr and increase free RPr, thereby causing repression. In mutants containing a defective sugar-specific Enzyme II, that sugar would not cause a depletion of phospho-HPr and therefore of phospho-RPr, and therefore that sugar would not exert PTS-mediated repression. The crr mutation would eliminate RPr and therefore allow growth. The above model proposed by Saier and Moczydlowski (1978) suggests that the regulatory role of the PTS derives from its role as a protein kinase system. The model is similar to that proposed by Postma and Roseman (1976) except that the latter authors suggested that RPr, which they also considered to be Enzyme III^{glc}, might act directly at the level of transcription. This suggestion was based on the fact that in E. coli high levels of cAMP

can alleviate PTS-mediated repression. However, in S. typhimurium cAMP does not reverse the repression (Postma and Roseman, 1976), and alternative mechanisms for the interaction between cAMP levels and the PTS have been proposed. For example, Saier (1977) proposed that adenylate cyclase was subject to positive control by phospho-RPr, i.e. phospho-RPr activated adenylate cyclase. This was in addition to the negative role of free RPr in the regulation of the non-PTS permeases. This model would allow for the activation of adenylate cyclase concomitant with the release of the non-PTS permeases from inhibition, resulting in the increased levels of cAMP and inducer required for the transcription of the operons specifiying the catabolic enzyme systems (Saier, 1977).

The second distinct mechanism for the regulation of sugar uptake involves repression by intracellular sugar-phosphates and may be mediated by the direct binding of the sugar phosphates to a regulatory site on the cytoplasmic face of the transport protein for the regulated sugar (Saier and Moczydlowski, 1978). This model has been proposed to account for the catabolite inhibition by glucose or glucose 6-phosphate of the continued utilization of various PTS sugars (McGinnis and Paigen, 1969, 1973; Amaral and Kornberg, 1975). Amaral and Kornberg (1975) found that E. coli cells induced or constitutive for the glucose uptake system were unable to grow on fructose in the presence of α-methyl-glucoside (αMG) because the cells were unable to take up fructose. Mutants able to take up fructose in the presence of αMG fell into two classes: (i) mutants which were unable to take up αMG (defective in Enzyme II^{glc}) and (ii) mutants with a normal Enzyme II^{glc} but which had a regulatory deficiency which was mapped by transduction to a site within the gene for Enzyme II^{fru}. Thus the latter mutation may have altered a regulatory site on the Enzyme II^{fru}. A variety of approaches suggested that glucose 6-phosphate was the molecule interacting with that regulatory site to exclude fructose from the cell. Examples include (i) mutations which restricted the catabolism of glucose 6-phosphate increased the degree of inhibition of fructose

uptake exerted by exogenously-added glucose, but genetic loss of Enzyme II^{glc} relieved the inhibition, (ii) the addition of glucose 6-phosphate to the growth medium of bacteria constitutive for the hexose-phosphate transport system resulted in inhibition of fructose utilization, which was relieved by the regulatory mutation in Enzyme II^{fru} but not by loss of Enzyme II^{glc} (see also Kornberg and Jones-Mortimer, 1977). The notion that glucose 6-phosphate could bind to the intracellular surface of Enzyme II proteins was supported by the finding that Enzyme II^{glc} could catalyze phosphate transfer between glucose 6-phosphate and glucose, as long as glucose was present extracellularly and the glucose 6-phosphate intracellularly (cited by Saier and Moczydlowski, 1978). However, it still remains to be established if glucose 6-phosphate inhibits by directly modulating the activities of the carbohydrate transport proteins (Saier and Moczydowski, 1978).

Intracellular sugar-phosphates also inhibit the uptake of several carbohydrates in Staphylococcus aureus and Bacillus subtilis (Saier and Simoni, 1976) and are also involved in the catabolite repression of certain enzymes in B subtilis by a mechanism which does not act at the level of inducer uptake (Lopez and Thoms, 1977).

The third mechanism for the regulation of sugar uptake, and for which there is the least evidence, supposes that the conformations of certain permease proteins are influenced by the transmembrane electrical potential. This potential, according to the chemiosmotic theory, may correspond to the energized membrane state of oxidative phosphorylation. In this case an enhanced cellular level of chemiosmotic energy may either depress or increase transport activity. Evidence for such a mechanism, based largely on studies with inhibitors and ATPase mutants, has been reviewed by Saier and Moczydlowski (1978) and is not considered further here.

Thus, in summary, there are at least three different levels at which the utilization of carbohydrate compounds can be controlled:

uptake exerted by exogenously-added glucose, but genetic loss of Enzyme II^{glc} relieved the inhibition, (ii) the addition of glucose 6-phosphate to the growth medium of bacteria constitutive for the hexose-phosphate transport system resulted in inhibition of fructose utilization, which was relieved by the regulatory mutation in Enzyme II^{fru} but not by loss of Enzyme II^{glc} (see also Kornberg and Jones-Mortimer, 1977). The notion that glucose 6-phosphate could bind to the intracellular surface of Enzyme II proteins was supported by the finding that Enzyme II^{glc} could catalyze phosphate transfer between glucose 6-phosphate and glucose, as long as glucose was present extracellularly and the glucose 6-phosphate intracellularly (cited by Saier and Moczydlowski, 1978). However, it still remains to be established if glucose 6-phosphate inhibits by directly modulating the activities of the carbohydrate transport proteins (Saier and Moczydowski, 1978).

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The third mechanism for the regulation of sugar uptake, and for which there is the least evidence, supposes that the conformations of certain permease proteins are influenced by the transmembrane electrical potential. This potential, according to the chemiosmotic theory, may correspond to the energized membrane state of oxidative phosphorylation. In this case an enhanced cellular level of chemiosmotic energy may either depress or increase transport activity. Evidence for such a mechanism, based largely on studies with inhibitors and ATPase mutants, has been reviewed by Saier and Moczydlowski (1978) and is not considered further here.

Thus, in summary, there are at least three different levels at which the utilization of carbohydrate compounds can be controlled:

regulation of uptake ('inducer exclusion'), regulation of activity ('catabolite inhibition'), and regulation of the induction of synthesis (transcription) of the catabolic systems ('catabolite repression'). These regulatory processes are not necessarily independent, e.g. exclusion of inducer would result in the repression of enzyme synthesis, and the biochemical relationships between the various phenomena ('glucose effect', 'diauxie', 'transient repression', 'catabolite repression', 'catabolite inhibition', 'inducer exclusion', 'PTS-mediated regulation') are not well-defined. Similarly, knowledge on the role of cAMP in the phenomena is also confused. However, it would seem likely that multiple controls on carbohydrate catabolism are normally operative.

The above studies were done with organisms which utilize the PTS for the transport of most carbohydrates, and it is apparent that proteins of the PTS play an important role in the regulation of carbohydrate catabolism. However, the PTS seems to be limited to organisms using the EMP pathway of carbohydrate catabolism which produces 2 moles of PEP per mole of glucose. Organisms using the ED or PP pathways would presumably have a deficiency of PEP if the PEP was used in the uptake of the sugars (Kornberg and Jones-Mortimer, 1977). This may explain, for example, why P. aeruginosa possesses a PTS system for fructose but not for glucose, as the organism is able to metabolize fructose partially by the EMP pathway (see Section 6.3). However, Pseudomonas does regulate its carbohydrate catabolism. P. aeruginosa grows better on succinate than glucose, and succinate exerts much stronger catabolite repression than glucose on the synthesis of enzymes required for the catabolism of many compounds (reviewed by Clarke and Ornston, 1975). In fact, growth on succinate or other tricarboxylic acid cycle intermediates results in the catabolite repression of glucose-metabolizing enzymes, including those responsible for the transport of glucose (Midgley and Dawes, 1973; Mukkada et al., 1973; Ng and Dawes, 1973). However, glucose exerts strong catabolite repression over mannitol dehydrogenase synthesis, an effect which is not reversed by exogenous cAMP (Siegel et al., 1977). However, it is likely that the cells were

unable to take up the added cAMP (Siegel et al., 1977). It is also not clear whether the data of Siegel et al. (1977) reflect catabolite repression as defined earlier, whereby metabolism of the repressing compound is required, or if they reflect inducer exclusion. However, Smyth and Clarke (1975) showed that amidase synthesis in P. aeruginosa was subject to strong catabolite repression by succinate, malate and acetate and to weak catabolite repression by glucose. This repression was not due to inducer exclusion since a mutant constitutive for amidasesynthesis was equally affected. However, the metabolism of acetate was necessary for it to exert repression. Exogenous cAMP reversed the catabolite repression exerted by glucose but had no effect on that exerted by succinate, but the possibility that succinate inhibited the uptake of cAMP was not discounted (Smyth and Clarke, 1975). The authors concluded that amidase synthesis was subject to control by catabolite repression exerted in a manner similar to that exerted on various catabolic enzymes in E. coli.

The experiments described in Section 7.2 were undertaken to obtain information on the regulation of carbohydrate catabolism in R. trifolii. They were initially motivated by the isolation of the glk mutant strains which exhibited a pleiotropic growth defect. An analogy to pts I or pts H mutants was suggested in that glucokinase in R. trifolii was thought to be functionally analogous to the PTS in enteric bacteria, i.e. to phosphorylate glucose. The isolation of the glk mutants also offered an opportunity to assess whether there was a requirement for the metabolism of glucose to at least glucose 6-phosphate for the regulation of carbohydrate catabolism by glucose in R. trifolii. Thus the control of carbohydrate catabolism in glucokinase-positive and glucokinase-negative strains was compared.

The description of the work done is divided into three major sections. However, the experiments in Sections 7.2.1 and 7.2.2 were done concomitantly and the prior reading of Section 7.2.2.1 may facilitate the understanding of Section 7.2.1. The strains used for the work, together with their relevant characteristics, are listed in Table 30.

Table 30 Strains used for the work described in Section 7.2

Strain	Parent	Characteristics	Source or Reference
7000		wild-type	
7008	7000	trp-4	Section
7009	7008	trp-4 glk-4	Section 6.2.4
7010	7009	trp-4 glk-4 Dul$^+$	Dulcitol-specific revertant of 7009 (Section 7.2.1.3)
7011	7010	trp-4 glk-4 Dul$^+$ (Dul + 2DG)$^+$	Isolate of 7010 able to grow on dulcitol + 2-deoxy-glucose (Section 7.2.1.5)
7012	7000	his-6	Section
7013	7012	his-6 glk-7	Section 6.2.4
7015	7012	his-6 glk-1 Sam$^-$	Section 6.2.4
7017	7012	his-6 sam-7	NTG mutagenesis (Section 7.2.1.2)
7021	7013	his-6 glk-7 sam-11	NTG mutagenesis (Section 7.2.1.2)
7026	7015	his-6 Glc$^+$ Sam$^-$	Glucose-positive, Sam-negative revertant of 7015 (Section 7.2.1.2)
7027	7015	his-6 Glc$^+$ Sam$^+$	Glucose-positive, Sam-positive revertant of 7015 (Section 7.2.1.2)
7029	7009	his-6 glk-4 Lac$^+$	Lactose-specific revertant of 7009 (Section 7.2.1.3)
7046	7015	his-6 glk-1 Glc$^-$ Sam$^+$	Glucose-negative Sam-positive revertant of 7015 (Section 7.2.1.2)
7083	7026	his-6 Glc$^+$ Sam$^-$ Gal$^-$	Spontaneous mutant of 7026 unable to grow on galactose (Section 7.2.1.2)
7084	7000	nal-1	Spontaneous fast-growing nalidixic acid-resistant mutant of strain 7000 (Section 7.2.3)
7085	7000	nal-2	Spontaneous slow-growing nalidixic acid-resistant mutant of strain 7000 (Section 7.2.3)

7.2 Results

7.2.1 Studies on the nature of the pleiotropic defect in the glucokinase-negative strains

7.2.1.1 Preliminary studies.- As described in Sections 6.2.3 and 6.2.4, the *glk* mutant strains were impaired in growth on glucose, cellobiose, maltose, trehalose, maltitol, lactose, lactulose, raffinose, sucrose and dulcitol (Glk-negative phenotype). Strain 7015 was also unable to grow on sorbitol, arabitol and mannitol (SAM-negative phenotype). The finding that all revertants of strain 7009 isolated on glucose were able to grow on all of the above carbon sources suggested that the mutation causing the lack of glucokinase was the primary effector of the pleiotropic growth defect. However, the pleiotropy was not caused by an intracellular accumulation of glucose through some non-specific mechanism because the strains also had very slow growth rates on dulcitol and lactulose whose metabolism does not generate glucose. The lack of an inhibitory effect of accumulated glucose was further confirmed by testing the effect of glucose on the growth of strain 7009 on some other carbohydrates. The presence of glucose in the medium had no effect on the growth of strain 7009 on fructose, galactose, inositol, mannitol or ribitol. (Further data on the effect of glucose and glucose analogues on the growth of wild-type and *glk* mutant strains on various carbohydrates are given in Section 7.2.1.5). The results thus suggested that the cause of the observed growth repression was less straightforward, and that glucokinase may play a regulatory role, possibly analogous to that of the PTS in PTS-mediated repression.

7.2.1.2 Further studies on strain 7015.- Strain 7015 was exceptional amongst the 40 glucose-negative isolates tested in that it was unable to grow on sorbitol, arabitol or mannitol. The strain, however, contained the relevant dehydrogenase activities when grown in RDM containing mannitol, glucose and glycerol (Section 6.2.4). This finding suggested that strain 7015 was deficient in the transport system(s) for the relevant polyols since it grew on fructose, the product of

mannitol and sorbitol dehydrogenases in R. meliloti at least (Martinez-de Drets and Arias, 1970). Furthermore, the finding suggested that the synthesis of the dehydrogenases was not repressed by glucose in strain 7015. It should be noted that the three dehydrogenase activities are subject to co-ordinate induction, are not separable by DEAE-cellulose chromatography and are inactivated by heat at the same rate, suggesting that they are the manifestations of a single enzyme in R. trifolii 7000 (Primrose, personal communication). The enzyme will henceforth be termed SAM dehydrogenase.

Transport studies confirmed that strain 7015 was unable to take up mannitol (Fig. 13). The fact that mannitol induced SAM dehydrogenase in strain 7015 suggests that either the strain was able to take up enough mannitol to induce the enzyme or that the enzyme was induced by extracellular mannitol. It was not determined which possibility was correct, but the fact that the strain had a tight phenotype supports the latter interpretation.

In order to determine whether the inability of strain 7015 to synthesize the mannitol transport system was associated with its inability to grow on glucose, revertants of strain 7015 were isolated and tested on each carbohydrate. The isolation of revertants also offered an opportunity to determine whether there were separate permeases for each polyol under co-ordinate control, or whether one permease was responsible for the uptake of the three polyols. If the former was the case, the isolation of revertants able to grow on only one of the polyols might be predicted. The results of the revertant analysis are presented in Table 31. The revertants isolated on glucose fell into two classes, viz. glucose-positive, SAM-positive (type-strain 7027) and glucose-positive SAM-negative (type-strain 7026), whereas all revertants isolated on any of the polyols were glucose-negative, SAM-positive (type-strain 7046). Note that all glucose-positive revertants were lactose and dulcitol positive, whereas all glucose-negative revertants were lactose and dulcitol negative. The failure to find glucose-positive, SAM-positive revertants amongst

Fig. 13 Mannitol uptake by strains 7012 and 7015. The bacteria were grown in RDM containing the specified carbon source(s) (each at 0.4% w/v) and were assayed for mannitol uptake as described in Section 2.11.

○, strain 7012 grown with mannitol plus inositol as carbon source

□, strain 7012 grown with inositol as carbon source

●, strain 7015 grown with mannitl plus inositol as carbon source

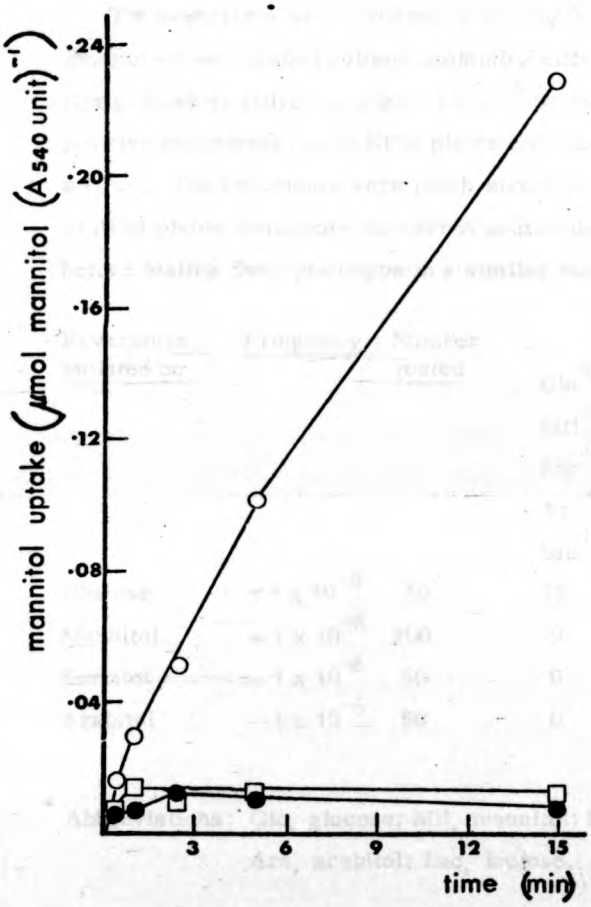

mannitol uptake (μmol mannitol (A540 unit)⁻¹)
·24
·20
·16
·12
·08
·04
3
6
9
12
15
time (min)

Table 31 Phenotypes of revertants of strain 7015

The revertants were isolated by plating 0.1 ml aliquots of an inositol-grown washed culture containing either ~ 10^9 bacteria ml^{-1} (for glucose-positive revertants) or ~ 10^8 bacteria ml^{-1} (for polyol-positive revertants) on to RDM plates containing the relevant carbon source. The revertants were patch-streaked with a toothpick on to RDM plates containing the carbon source used for their isolation before testing their phenotype in a similar manner.

Revertants isolated on:	Frequency	Number tested	Phenotype*		
			Glc^+ Mtl^+ Sor^+ Ara^+ Lac^+	Glc^- Mtl^+ Sor^+ Ara^+ Lac^-	Glc^+ Mtl^- Sor^- Ara^- Lac^+
Glucose	$< 1 \times 10^{-8}$	50	13		37
Mannitol	$\sim 1 \times 10^{-6}$	200	0	200	
Sorbitol	$\sim 1 \times 10^{-6}$	50	0	50	
Arabitol	$\sim 1 \times 10^{-6}$	50	0	50	

* Abbreviations: Glc, glucose; Mtl, mannitol; Sor, sorbitol; Ara, arabitol; Lac, lactose.

the revertants isolated on the polyols may reflect the fact that revertants isolated on the polyols arose at a frequency more than 100-fold higher than those isolated on glucose. The failure to find revertants able to grow on only one of the polyols suggests, but does not prove, that the polyols are taken up by a single transport system.

The finding of the two classes of glucose-positive revertants suggested that a single regulatory mutation was responsible for both the Glk^- and Sam^- phenotypes. However, during routine subculturing, a single colony of strain 7026 streaked from a RDM-glucose plate on to a RDM-galactose plate was found to be galactose-negative (type-strain 7083) unlike strain 7015. Further single colonies of strain 7026 grown on RDM-glucose plates were then tested on RDM-galactose plates and approximately 10% were galactose-negative. All were fructose-positive. Glucose-positive revertants of strain 7013 were then tested on galactose but all were galactose-positive. Further glucose-positive revertants of strain 7015 were then isolated and tested on galactose and mannitol (Table 32). The results were confusing and suggested that strain 7015 was genetically unstable.

Table 32 Phenotypes of glucose-positive revertants of strain 7015

	Phenotype				Total No.
	Gal^+Mtl^+	Gal^-Mtl^+	Gal^+Mtl^-	Gal^-Mtl^-	
Number	8	3	41	7	59

This was further confirmed by the finding that 5 of 100 mannitol-positive revertants of strain 7015 were galactose-negative and 3 of 100 inositol-grown colonies of strain 7015 were galactose-negative. The galactose-negative mutants of strain 7015 thus obtained reverted to a galactose-positive phenotype at a frequency of 10^{-6}-10^{-7}. The isolation of spontaneous galactose-negative mutants was unique to strain 7015; no similar mutants (visible as microcolonies on RDM-galactose plates) were noticed after spreading diluted inositol-grown

cultures of strains 7009 and 7013 for single colonies on RDM-galactose plates (20 plates, each containing 50-100 colonies, of each strain were visually scanned). Thus it was concluded that strain 7015 was exceptionally genetically-unstable and therefore unsuitable for future work.

Because of the above findings it was decided to attempt to isolate further glucose-negative mutants of strain 7012 and mannitol-negative mutants of strains 7012 and 7013. Sam-negative mutants of strain 7013 were to be used to test whether any glucose-positive revertants suppressed the Sam-negative phenotype, whereas the mutants of strain 7012 were isolated in the hope of finding glucose-negative, Sam-negative mutants similar to strain 7015. The isolation of Sam-negative mutants would also serve a more general purpose. It was hoped to isolate Sam dehydrogenase-deficient rather than permease-deficient mutants and also to isolate mutants deficient in the synthesis of all inducible carbohydrate-catabolic enzyme systems, i.e. mutants analogous to cya or crp mutants of E. coli (see Perlman and Pastan, 1969; Kumar, 1976). The former class of mutants would be useful for studying the number of enzymes involved in the SAM dehydrogenase activities and possibly the induction of these enzymes, whereas the latter class would be useful for studying the general control of inducible enzyme synthesis in R. trifolii.

As stated earlier (Section 6.2.2), attempts to isolate further glucose-negative mutants were unsuccessful, but mannitol-negative mutants were relatively easily isolated (Table 33). Each mutagenized culture from four independent experiments was subjected to one ampicillin enrichment cycle and the mutants were isolated as micro-colonies on

Table 33 Isolation of mannitol-negative mutants of strains 7012 and 7013

Cultures were mutagenized with NTG as described in Section

Strain	Non-selective medium	Selective medium	No. of mutants isolated	Allele numbers	Type-strains
7012	GSYC	RDM-mannitol	2	sam-2, sam-3	-
7012	RDM-fructose	RDM-mannitol	7	sam-4 to sam-10	7017
7013	RDM-fructose	RDM-mannitol	5	sam-11 to sam-15	7021
7013	RDM-inositol	RDM-mannitol	2	sam-16 to sam-17	-

RDM-mannitol plates. Each mannitol-negative mutant displayed a Sam-negative, ribitol and inositol positive phenotype. All Sam-negative mutants derived from strain 7012 were glucose-positive. All of the Sam-negative mutants isolated from 7012 and 7013 were unable to take up mannitol after growth in RDM-broth containing mannitol plus inositol (see Table 34 for representative results). The uptake of mannitol by the revertants isolated from strain 7015 was also tested at the time these experiments were done. Both mannitol-positive revertants of strain 7015 tested (strains 7027 and 7046) took up mannitol only when induced, i.e. the SAM transport system in the revertants was under similar control as in the wild-type strain 7012 (Table 34). No further work was done with strain 7015 or its revertants; therefore it is not known whether the mutation causing the Sam-negative phenotype in strain 7015 was in a structural or regulatory gene. If revertants synthesizing the SAM transport system constitutively had been obtained, this would have provided evidence for an independent origin of Glk^- and Sam^- phenotypes in strain 7015.

None of the 50 glucose-positive revertants of strain 7021 tested regained the Sam-positive phenotype. Therefore it was concluded from the above work that the induction of the SAM transport system was probably not under the same control as that causing the Glk^- phenotype in strain 7015.

7.2.1.3 Revertants of strains 7009 and 7013. - Revertants were isolated either by plating aliquots of mannitol-grown washed cultures or by streaking single colonies from RDM-mannitol plates on to RDM plates containing the appropriate carbon source. The latter method was generally preferred because it gave a cleaner background. Strain 7009 was used for most of this work.

As already stated, revertants of 7009 isolated on glucose were able to grow on all the sugars (Glk^+ phenotype), indicating both that the pleiotropy was due to a single point mutation and that the defect causing the inability to grow on glucose was the primary cause of the Glk^- phenotype (Section 6.2.4). Revertants isolated on most of the

Table 34 Accumulation of ^{14}C-labelled mannitol by wild-type and Sam-negative strains

Bacteria suspended off RDM-inositol plates were inoculated into 250 ml Erlenmeyer flasks containing 100 ml of RDM containing 0.4% inositol and 0.4% mannitol, unless otherwise stated,* grown overnight and harvested in the exponential phase of growth. Uptake experiments were done as described in Section 2.11 except that the specific activity of the ^{14}C-labelled mannitol was 0.62 μCi μmol^{-1}. Samples were taken 1 minute and 20 minutes after the addition of the labelled mannitol. Results are expressed as cpm (A_{540} unit)$^{-1}$.

Strain	Sample time 1 minute	Sample time 20 minutes	Δ cpm min^{-1} (A_{540} unit)$^{-1}$
7012	3311	31643	1491
7012*	1234	3268	107
7015	849	1176	17
7012 sam-1	930	1295	19
7012 sam-2	868	1100	12
7012 sam-7	1060	977	-4
7013 sam-11	977	994	1
7013 sam-15	876	1679	42
7013 sam-16	1013	1438	22
7013 sam-17	844	985	7
7026	804	969	9
7027	3186	21755	977
7027*	935	1935	53
7046	3642	34491	1624
7046*	1010	3485	130

* Cultures were grown in RDM containing inositol as sole carbon source

other sugars fell into two classes: those that regained the ability to utilize for growth only the sugar contained in the medium upon which they were isolated (sugar-specific) and those that were Glk^+ (Table 35). Sugar-specific revertants isolated on either lactose or lactulose-containing medium were able to utilize both carbon sources. It was also found, in the case of dulcitol, lactose and lactulose, that the inclusion of 2-deoxyglucose (2DG) in the medium used for isolating the revertants gave a cleaner background while allowing the revertants to grow slowly. When purified, these revertants were inhibited to a similar extent as wild-type (see Section 7.2.1.5) when grown on medium containing the relevant sugar plus 2DG. The inclusion of 2DG in medium containing dulcitol resulted in 100% of dulcitol-specific revertants, and increased the proportion of lactose-specific revertants isolated on medium containing lactose (Table 35). It is worth noting that in no case were specific glucose-positive or glucose-negative, lactose plus dulcitol positive revertants found.

One dulcitol-specific (strain 7010) and one lactose-specific (strain 7029) revertant of strain 7009 was chosen for further study. Strain 7010 grew at a faster rate on dulcitol, ribitol and xylitol than strains 7008 or 7009 (Table 36), indicating that the dulcitol-specific mutation in strain 7010 overcame the block causing the slow growth of the wild type strain on the three polyols. Strain 7029 also grew faster (doubling time 5.7 h) than strain 7008 (doubling time 6.2 h) on lactose. Glucose-positive revertants of strain 7010 grew on the polyols at the rate characteristic of strain 7010, indicating that the defect causing the Glk^- phenotype was not directly associated with the causation of the faster growth rate of strain 7010 on the three polyols.

7.2.1.4 Heat stability of glucokinase in glucokinase-positive revertants of strain 7009. - Since the revertant analysis indicated that the mutation causing the inability to grow on glucose was the primary effector of the pleiotropic phenotype, it was of interest to determine whether the mutation was in a structural or regulatory gene for glucokinase. Attempts to isolate temperature sensitive glucose-positive revertants from strains 7009 and 7013, able to grow in

Table 35 Phenotypes** of revertants of strains 7009 and 7013

Isolated on:	Parent strain	Glk+ phenotype (%)	Sugar--specific phenotype (%)	Total No. tested
Glucose	7009	100	0	400
Maltose	7009	100	0	11
Cellobiose	7009	100	0	7
Sucrose	7009	93	7	54
Lactose	7009	74	26	76
Lactose + 2DG*	7009	38	62	100
Lactulose + 2DG	7009	30	70	110
Dulcitol	7009	33	67	124
Dulcitol + 2DG	7009	0	100	200
Glucose	7013	100	0	74
Dulcitol + 2DG	7013	0	100	300

*Abbreviation: 2DG, 2-deoxyglucose (0.4% w/v)

**Each revertant was tested on glucose, sucrose, lactose, lactulose and dulcitol.

Table 36 Doubling time of strains 7008, 7009 and 7010 containing dulcitol, xylitol or ribitol as sole carbon source

Doubling times (h) are the average of two determinations.

Carbon source	Strain 7008	7009	7010
dulcitol	9.0	16.4	5.2
ribitol	6.4	6.2	4.8
xylitol	14.6	13.6	5.4

glucose at 14° C but not at 28° C, were unsuccessful. Therefore it was decided to determine the heat stability of glucokinase in glucose-positive revertants.

As stated earlier (Section 6.2.4), it was found that many glucose-positive revertants did not regain glucokinase activity. However, ten revertants of strain 7009 which did regain glucokinase were isolated and the glucokinase in them tested. Glucokinase from strain 7008 was a relatively heat-stable enzyme (Fig. 14a, b) and so extracts of the glucokinase-positive revertants were treated at 54° C for 15 min. In each case, the extracts still contained appreciable amounts of glucokinase activity after this treatment. Therefore no evidence for the glk mutation having occurred in the structural gene for glucokinase was found.

7.2.1.5 The effect of 2-deoxyglucose on the growth of wild-type, mutant and revertant strains. The use of the glucose analogues 2DG and αMG has proved useful for defining systems of glucose uptake in P. aeruginosa (Midgley and Dawes, 1973; Mukkada et al., 1973) and enteric bacteria (review by Postma and Roseman, 1976). They have also been used for defining some of those carbohydrates whose utilization is susceptible to control via PTS-mediated repression and catabolite inhibition in enteric bacteria (e.g. Amaral and Kornberg, 1975; Saier and Roseman, 1976a).

The effect of 2DG on carbohydrate utilization by wild-type, Glk⁻ and sugar-specific revertant strains is shown in Table 37. The inclusion of 2DG in the medium had no effect on the growth of any of the strains on most carbohydrates. However, it was found that 2DG inhibited the growth of strain 7008 on those carbon sources that strain 7009 was unable to grow on, except glucose and sucrose. Also 2DG inhibited the growth of all strains on ribitol and xylitol. The fact that the glk mutant was unable to grow on ribitol or xylitol plus 2DG suggested that phosphorylation of the glucose analogue was not required for the inhibitory effect to be exerted. Also, the fact

Fig. 14 Heat stability of glucokinase in extracts of strain 7008. Heat treatments were done on 1 ml samples of extract contained in 25 ml Erlenmeyer flasks. The extracts were heated to the desired temperature by gently swirling the flasks in a water-bath set at that temperature. After treatment, the extracts were cooled by plunging the flasks into an ice-salt bath and gently swirling them. The extracts were then centrifuged at full-speed in a micro-centrifuge to remove precipitated protein and the cleared supernatant assayed for glucokinase.

(a) Time-course of glucokinase inactivation at 49° C

(b) Temperature-curve of glucokinase inactivation. Extracts were held at the specified temperature for 15 min.

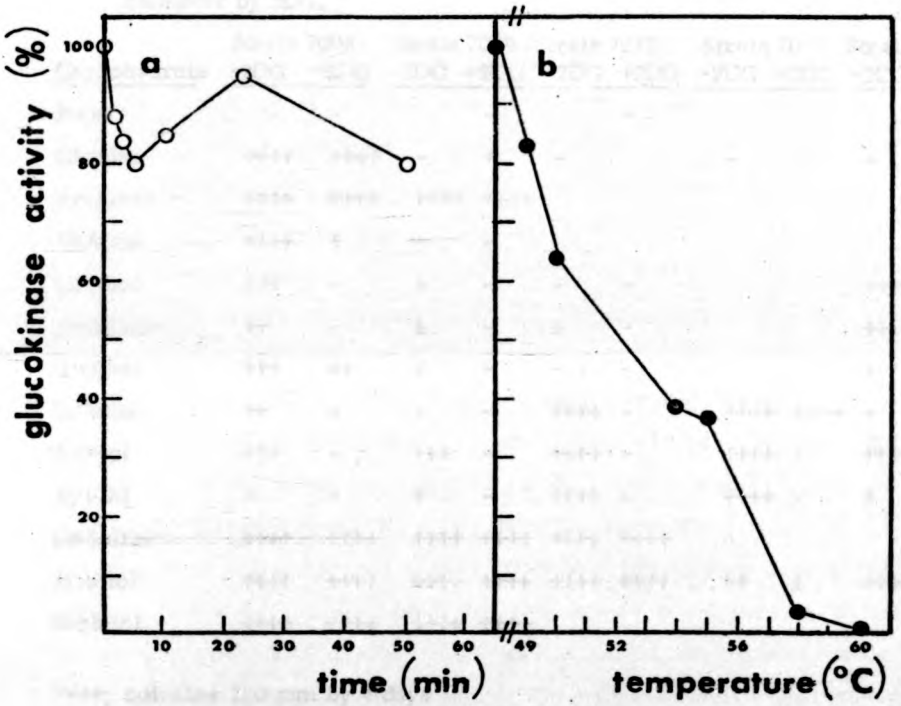

glucokinase activity (%)
100
80
60
40
20
a
b
10
20
30
40
50
60
49
52
56
60
time (min)
temperature (°C)

Table37 The effect of 2-deoxyglucose on the growth of strains 7008, 7009, 7010 and 7029 on various carbohydrates

Single colonies of the strains were streaked from RDM-inositol plates on to RDM plates containing the specified carbon source (0.4% w/v) and 2-deoxyglucose (2DG) (0.4% w/v) where specified. The diameter of the single colonies obtained was measured. However, it is worth noting that growth on the main streaks was affected similarly to single-colony diameter by 2DG.

	Strain 7008		Strain 7009		Strain 7010		Strain 7011		Strain 7029	
Carbohydrate	-2DG	+2DG	-2DG	+2DG	-2DG	+2DG	-2DG	+2DG	-2DG	+2DG
None		-		-		-				
Glucose	++++	++++	-	-	-		-		-	
Fructose	++++	++++	++++	++++						
Maltose	++++	+	-	-						
Lactose	+++	-	-	-	-	-			+++	-
Lactulose	++	-	±	-	±	-			++	-
Sucrose	+++	++	-	-	-	-			-	-
Dulcitol	++	-	-	-	++++	-	++++	++++	-	-
Ribitol	+++	-	+++	-	++++	-	++++	-	+++	-
Xylitol	+	-	+	-	++++	-	++++	-	+	-
Mannitol	++++	++++	++++	++++	++++	++++				
Inositol	++++	++++	++++	++++	++++	++++	+	±	+++	++
Sorbitol	++++	++++	++++	++++						

++++, colonies 2.0 mm by 4 days

+++, colonies 2.0 mm by 5 days

++, colonies 1.0 mm by 5 days

+, colonies 1.0 mm by 6 days

±, colonies 0.4 mm by 6 days

-, colonies < 0.2 mm by 6 days

that the sugar-specific revertants of strain 7009 were still sensitive to inhibition by 2DG (Table 37) provided further evidence that phosphorylation of 2DG was unnecessary, and also indicated that the inhibition caused by 2DG operated by a separate mechanism to that resulting from the glk mutation.

The effect of 2DG did not parallel the effect of glucose on the glk strains. Strain 7009 grew on ribitol in the presence of glucose as did strains 7010 and 7029 on dulcitol or lactose respectively. Nevertheless, in all three cases the presence of glucose in the medium reduced the growth-rate by approximately 10-30%, and it seems reasonable to assume that 2DG was acting as an analogue of glucose. The lack of inhibition of the growth of strain 7008 on glucose by 2DG would be explained if glucose competitively inhibited 2DG uptake and caused an exit of 2DG from the cells as occurs in P. aeruginosa wherein 2DG is taken up by the glucose transport system (Mukkada et al., 1973). Also, a more severe effect by the non-metabolizable 2DG than by glucose might be expected since glucose is probably subject to transformations other than phosphorylation, such as those involved in polysaccharide formation. A further possible reason for the greater severity of the effect exerted by 2DG would be if the bacteria accumulated 2DG to a higher intracellular level than they accumulated glucose. Support for the suggestion that 2DG was acting as a metabolic analogue of glucose came from the finding that 2DG inhibited the growth of strain 7008 only on those of the polyols whose utilization was subject to catabolite repression by glucose (see Section 7.2.2.1).

The finding that 2DG inhibited growth of strain 7010 on dulcitol provides an explanation for the finding that only dulcitol-specific revertants were found when strain 7009 was streaked on RDM plates containing dulcitol plus 2DG. These revertants arose as small colonies against a clean background after extended (10 days) incubation of the plates. The background was very clean because dulcitol utilization in strain 7009 was subject to both types of inhibition (i.e. due to the glk mutation and to 2DG) and the dulcitol-specific revertants had a selective advantage because of their faster growth rate on

dulcitol. One isolate of strain 7010 was found which was able to grow rapidly on dulcitol in the presence of 2DG. This isolate, labelled strain 7011, arose as a large mucoid derivative on a RDM plate containing dulcitol plus 2DG which had been heavily streaked with strain 7010. Strain 7011 was the only such isolate found from several such streaked plates of strains 7009 and also 7008, i.e. strain 7011 was the only isolate ever obtained that grew well on dulcitol plus 2DG. Strain 7011 was found to be trp$^-$ and Trp$^+$ revertants of it nodulated clover, clearly indicating that it was not a contaminant.

7.2.1.6 Enzyme activities in strains 7008, 7009, 7010 and 7011 cultured in the presence of dulcitol and/or other polyols. - In an attempt to determine the basis for the impaired growth rate of strain 7009 on dulcitol, the induction and amounts of various enzymes resulting from growth in the presence of dulcitol of strains 7008, 7009, 7010 and 7011 were determined.

The enzyme activities found in extracts prepared from strain 7008 grown in various media are given in Table 38 . Growth of strain 7008 in RDM containing dulcitol resulted in the induction of three polyol dehydrogenase activities dependent upon dulcitol, ribitol and xylitol. No other polyol dehydrogenases were induced. In order to determine whether one enzyme was responsible for the three activities induced, or alternatively whether two or more enzymes were induced, strain 7008 was grown on ribitol. The growth of strain 7008 on ribitol resulted in the induction of ribitol and SAM dehydrogenase activities but not of dulcitol- or xylitol-dependent activities. This lack of reciprocality suggested that dulcitol dehydrogenase displayed ribitol-dependent activity but was induced only by dulcitol. However, evidence obtained with strain 7010 grown on dulcitol and on ribitol (see below) suggested that the dulcitol- and ribitol-dependent activities induced by growth on dulcitol were the results of separate enzymes. The evidence suggested that growth of strain 7008 on dulcitol induced both

Table 38 The activities of various enzymes in cell-free extracts prepared from strain 7008 grown in RDM containing various carbon sources

Bacteria were grown in 1 litre Erlenmeyer flasks containing 500 ml RDM plus the specified carbon sources, each at 0.4% (w/v). The flasks were inoculated with bacteria suspended off RDM-mannitol plates. The cultures were harvested, extracts prepared and enzymes assayed as described in Chapter 2. The enzyme activities are expressed as nmol min^{-1} (mg protein)$^{-1}$. Each value is the average of those obtained from two independent experiments.

	Enzyme activities*						
Carbon source(s)	DDH	RDH	XDH	MDH	SDH	IDH	GK
Dulcitol	250	272	54	38	25	58	223
Ribitol	7	96	16	219	67	49	200
Dulcitol + Inositol	155	172	39	33	24	678	207
Dulcitol + Inositol + Glucose	21	26	17	24	14	438	245
Dulcitol + Glucose	25	23	16	31	24	44	212
Dulcitol + Fructose	59	58	39	40	23	55	194
Inositol	15	15	13	26	20	742	257

*Abbreviations: DDH, dulcitol dehydrogenase; RDH, ribitol dehydrogenase; XDH, xylitol dehydrogenase; MDH, mannitol dehydrogenase; SDH, sorbitol dehydrogenase; IDH, inositol dehydrogenase; GK, glucokinase.

dehydrogenases whereas growth on ribitol induced ribitol but not dulcitol dehydrogenase. This suggests that the induction of the various dehydrogenases by the polyols is very complex, especially since ribitol induced SAM dehydrogenase whereas dulcitol did not. It was not determined whether xylitol was a substrate of dulcitol dehydrogenase or whether xylitol dehydrogenase was a separate enzyme.

When strain 7008 was grown in RDM containing inositol in addition to dulcitol, inositol dehydrogenase activity was induced, and the other dehydrogenase activities were repressed by 34-42%. If glucose was included in the above medium, neither dulcitol, ribitol nor xylitol dehydrogenase activities were induced above the basal level, and the amount of inositol dehydrogenase activity was reduced by 40%. Growth of the bacteria in medium containing dulcitol plus glucose resulted in similar basal amounts of dulcitol, ribitol and xylitol dependent activities, showing that glucose strongly repressed the induction of these activities. If the medium contained dulcitol plus fructose rather than dulcitol plus glucose, higher amounts of the activities were obtained, but they were still less than 25% of those obtained when dulcitol was the sole carbon source. Glucokinase was present in fully-induced amounts irrespective of the carbon source(s) present in the medium (Table 38).

The growth of strain 7009 in RDM containing dulcitol plus inositol resulted only in the induction of inositol dehydrogenase. The amount of this enzyme was reduced by approximately 15% when glucose was included in the medium. Neither dulcitol, ribitol nor xylitol dehydrogenase activities were induced by growth of strain 7009 in these media (Table 39). However, when strain 7009 was grown with dulcitol as sole carbon source and harvested after 72 h growth, fully-induced amounts of dulcitol, ribitol and xylitol dehydrogenase activities were obtained (Table 39). In no case was glucokinase present, and samples of the culture taken just prior to harvesting still seemed to display the glucose-negative, dulcitol-

Table 39 The activities of various enzymes in cell-free extracts prepared from strain 7009 grown in RDM containing various carbon sources

See legends and footnote to Table 38 for conditions and abbreviations used.

	Enzyme activities						
Carbon source(s)	DDH	RDH	XDH	MDH	SDH	IDH	GK
Dulcitol*	249	287	68	-**	-	49	0
Ribitol	7	88	11	163	93	-	0
Dulcitol + Inositol	8	10	6	-	-	613	0
Dulcitol + Inositol + Glucose	5	8	10	-	-	528	0
Inositol	5	10	10	-	-	618	0

* Bacteria were grown for 72 h before harvesting in this case only.

** -, not determined.

negative phenotype of strain 7009. It was not possible to grow strain 7009 in RDM containing dulcitol plus glucose because such cultures were found to contain a high percentage of dulcitol-specific revertants. Nevertheless, the results in Table 39 suggested that in strain 7009 the induction of the enzyme(s) responsible for the dulcitol, ribitol and xylitol dehydrogenase activities was hypersensitive to repression by the second carbon source, i.e. inositol. Support for this hypothesis was obtained by growing strain 7009 in RDM containing dulcitol (0.4% w/v) plus the following second carbon sources: inositol (0.05% w/v), glycerol (0.1% v/v) or galactose (0.05%). In no case was dulcitol, ribitol or xylitol dehydrogenase activity found in extracts prepared from strain 7009 grown in any of the above media. It is worth noting that when strain 7009 was grown with ribitol as sole carbon source, the same spectrum of enzyme activities was found as was found in strain 7008 under the same conditions, i.e. ribitol dehydrogenase was induced (compare data in Tables 38 and 39).

The activities of various enzymes in extracts prepared from strain 7010 grown in media containing the same carbon sources used for strain 7008 (Table 38) are given in Table 40 . The dulcitol,

Table 40 The activities of various enzymes in cell-free extracts prepared from strain 7010 grown in RDM containing various carbon sources

See legend and footnote to Table 38 for conditions and abbreviations used.

	Enzyme activities						
Carbon source(s)	DDH	RDH	XDH	MDH	SDH	IDH	GK
Dulcitol	275	314	161	17	68	56	0
Ribitol	261	282	139	156	142	-	0
Dulcitol + Inositol	175	188	119	14	37	407	-
Dulcitol + Inositol + Glucose	254	275	112	30	46	384	0
Dulcitol + Glucose	280	298	119	26	56	66	0
Dulcitol + Fructose	195	212	116	21	60	73	-
Inositol	307	356	133	18	51	577	0

ribitol and xylitol dehydrogenase activities were expressed constitutively in strain 7010. They were also insensitive to repression by glucose and less sensitive to repression by fructose. The repression obtained with fructose in three independent experiments was in the range 30-35%. A maximal 36% repression of dulcitol dehydrogenase activity by inositol was also observed, as shown in Table 40 , but this repression was more variable and its significance was difficult to judge. Also the repression exerted by inositol seemed to be dependent upon the presence of dulcitol in the medium although the strain was constitutive for dulcitol dehydrogenase.

The data in Table 40 also suggest that the dulcitol and ribitol dehydrogenase activities induced by dulcitol were the result of separate enzymes. The mean of the ratio of dulcitol dehydrogenase activity to ribitol dehydrogenase activity was 0.91 ± 0.03 for the experiments listed in Table 40 , i.e. this ratio was not altered by the growth of strain 7010 on ribitol. Therefore, no extra ribitol dehydrogenase activity was induced, although the SAM dehydrogenase activity, normally obtained by growth on ribitol, was induced.

It was also found that the addition of dulcitol to the ribitol dehydrogenase assay mixture gave an increased rate of NAD^+ reduction, although the increase obtained was only approximately 30%. Nevertheless, both these observations suggest that dulcitol induced both dulcitol dehydrogenase and ribitol dehydrogenase. The only alternative explanation is that a ribitol dehydrogenase normally induced by growth on ribitol was not induced under such conditions in strain 7010. This seems unlikely, especially since the SAM dehydrogenase activity normally induced by ribitol was induced by ribitol in strain 7010.

The finding that the dulcitol-specific revertants of strain 7009 synthesized dulcitol dehydrogenase constitutively suggested that the Glk^- phenotype was manifested through inducer exclusion. It is also worth noting that the amount of ribitol dehydrogenase in strain 7010 was approximately three-fold that found in strain 7008 grown on ribitol; this may explain the faster growth of strain 7010 on ribitol compared to strain 7008. However, strain 7010 also grew much faster than 7008 in dulcitol, although dulcitol dehydrogenase was induced to a similar level in both strains.

Strain 7011, which was isolated from strain 7010, grew on dulcitol in the presence of 2DG, unlike strain 7010. The enzyme activities found in extracts prepared from strain 7011 grown in various media are shown in Table 41. The results differ in two surprising respects from those found for strain 7010. Firstly, strain 7011 was constitutive for the SAM dehydrogenase activities and secondly the inositol dehydrogenase in the strain seemed to be regulated differently than in the other strains. Even when strain 7011 was cultured with inositol as sole carbon source, inositol dehydrogenase was only present at approximately half the level found under the same conditions in the other three strains examined, and when dulcitol was included in medium together with inositol, the enzyme was repressed to only one-third of the level found on inositol alone. Therefore the growth of strain 7011 on RDM-inositol plates was examined. In contrast to the

Table 41 The activities of various enzymes in cell-free extracts prepared from strain 7011 grown in RDM containing various carbon sources

See legend and footnote to Table 38 for conditions and abbreviations used.

	Enzyme activities						
Carbon source(s)	DDH	RDH	XDH	MDH	SDH	IDH	GK
Dulcitol	324	360	200	684	272	37	0
Dulcitol + Inositol	300	323	175	629	309	113	0
Dulcitol + Inositol + Glucose	282	320	170	588	253	88	0
Inositol	275	302	167	637	226	339	0

other strains, 7011 grew poorly on inositol (colony diameter ~ 1 mm by 5 days) and furthermore, the inclusion of 2DG in the medium together with inositol completely inhibited growth (Table 37). The effect of 2DG on the growth of strain 7011 on the polyols other than dulcitol and inositol was similar to that shown for strain 7010 in Table 37 . Therefore, the mutation(s) by which strain 7011 was derived from strain 7010 caused at least the following phenotypic effects: ability to grow on dulcitol in the presence of 2DG; constitutive synthesis of the enzyme(s) responsible for SAM dehydrogenase activities; sensitivity of inositol dehydrogenase to catabolite repression by dulcitol; poor growth on inositol; sensitivity to inhibition by 2DG of growth on inositol. Thus strain 7011 was a very peculiar strain. However, whether all the phenotypic effects were due to a single mutation was unknown, but if they were, the causal mutation must occur very infrequently since strain 7011 was the only strain found able to grow on dulcitol plus 2-deoxyglucose. The phenotype on plates containing dulcitol plus deoxyglucose was very apparent, and so any other such mutants should have been easily observed. The fact that they were not suggests that strain 7011 differs from strain 7010 by more than one mutation.

7.2.1.7 Enzyme activities in wild-type, mutant and revertant strains cultured in the presence of lactose.- In order to investigate the basis for the defective growth of strain 7009 found when lactose was the sole carbon source present in the medium, experiments comparable to those described in the preceding section, but using lactose in the place of dulcitol, were done with strains 7008, 7009 and 7029. In the initial experiments, the activity of β-galactosidase was measured by following the hydrolysis of ortho-nitrophenyl-β-D-galactopyranoside (ONPG). However, it was later found that paranitro phenyl-β-D-galactopyranoside (PNPG) was hydrolyzed at a four-fold faster rate than ONPG by the extracts, as was reported for β-galactosidase from R. meliloti (Niel et al., 1977). In some experiments lactose hydrolysis was also measured by coupling the formation of galactose from lactose to $NADP^+$ reduction via the galactose dehydrogenase which all the extracts contained. The amount of β-galactosidase activity obtained using this assay was similar to that obtained by measuring ONPG hydrolysis.

β-galactosidase was a constitutive enzyme in strains 7008, 7009 and 7029. The presence of lactose in the medium did not cause an increase in the amount of β-galactosidase activity and the enzyme was not sensitive to repression by glucose (Table 42). Therefore the amount of β-galactosidase was not a primary factor causing the impairment of growth of strain 7009 on lactose.

However, there was no proof that β-galactosidase was the physiologically important enzyme involved in lactose catabolism in R. trifolii strain 7000. Nevertheless attempts to find alternative pathways were unsuccessful. ONPG-6-phosphate was not hydrolyzed by the extracts, suggesting that lactose was not phosphorylated prior to hydrolysis. Similarly, 2-ketolactose was not formed, indicating the absence of a pathway such as that found in A. tumefaciens (Bennaerts and De Ley, 1963). Also, strain 7009 did not grow on RDM-lactose plates and only grew very slowly in RDM-lactose broth (Fig. 5b). The amount of β-galactosidase activity in strain 7009

Table 42 β-galactosidase activity in cell-free extracts prepared from strains 7008, 7009 and 7029 grown in RDM containing various carbon sources

The flasks were inoculated, cultures harvested, and extracts prepared as described in the legend to Table 38 . β-galactosidase was measured by following PNPG hydrolysis, and the values reported are fourfold-higher than those obtained by measuring ONPG hydrolysis. The activities represent nmol. PNPG hydrolyzed min^{-1} $(mg\ protein)^{-1}$.

	Strain		
Carbon source(s)	7008	7009	7029
Lactose	119	-*	113
Lactose + Inositol	125	111	106
Lactose + Inositol + Glucose	93	94	102
Inositol	119	128	94

*not determined

(20-30 nmol min^{-1} $(mg\ protein)^{-1}$ when assayed either by ONPG hydrolysis or by coupling lactose hydrolysis to galactose dehydrogenase) would seem sufficient to support a faster growth-rate than that observed, if strain 7009 was able to transport lactose.

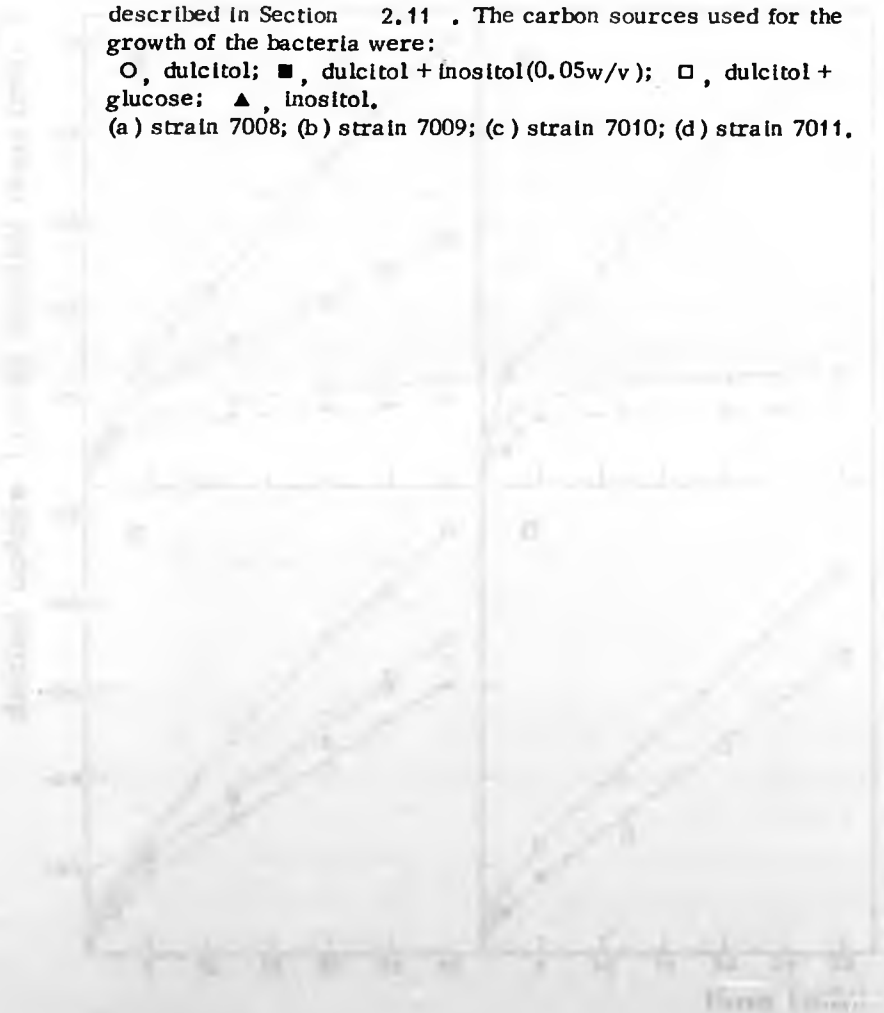
7.2.1.8 The transport of dulcitol by strains 7008, 7009, 7010 and 7011.

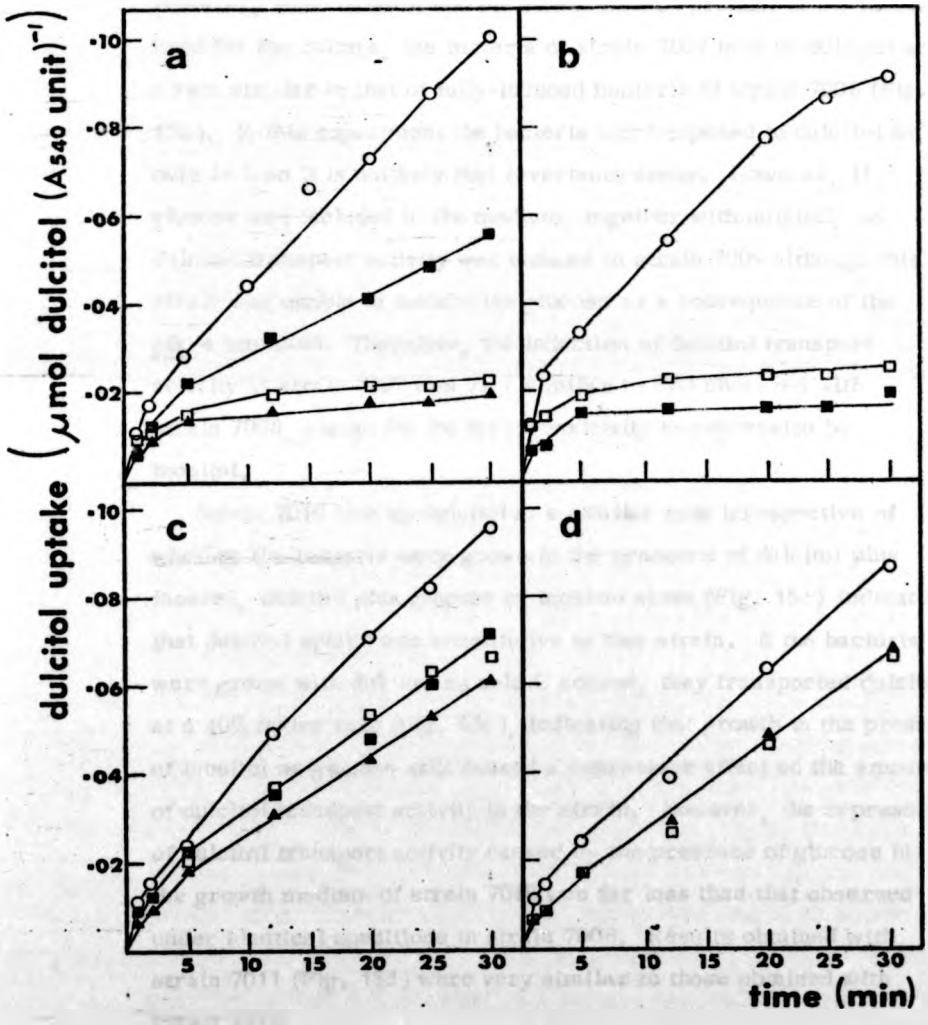
As described in Section 7.2.1.6, the growth of strain 7009 in RDM containing dulcitol plus inositol did not induce dulcitol dehydrogenase. Also, dulcitol-specific revertants of strain 7009 were constitutive for dulcitol dehydrogenase. These observations suggested that the failure of strain 7009 to grow on dulcitol may be due to failure to transport dulcitol. Therefore, a comparative study of dulcitol transport in strains 7008-7011 was undertaken.

Dulcitol transport by strain 7008 was both inducible and repressible (Fig. 15a). Bacteria fully-induced by growth with dulcitol as sole C source took up ^{14}C-labelled dulcitol, albeit relatively slowly. This rate was reduced by approximately 40% if inositol (0.05% or 0.4%) was included with dulcitol in the growth medium. If the growth medium contained glucose as well as

Fig. 15 Dulcitol transport by strains 7008, 7009, 7010 and 7011

Bacteria were grown in 1 litre Erlenmeyer flasks containing 500 ml RDM plus the specified carbon sources, each at 0.4% (w/v) unless otherwise specified. The flasks were inoculated with bacteria suspended off RDM-mannitol plates. The cultures were harvested after 16 h incubation and were assayed for dulcitol transport as described in Section 2.11 . The carbon sources used for the growth of the bacteria were:
O, dulcitol; ■, dulcitol + inositol (0.05w/v); □, dulcitol + glucose; ▲, inositol.
(a) strain 7008; (b) strain 7009; (c) strain 7010; (d) strain 7011.




dulcitol uptake (μmol dulcitol (A540 unit)⁻¹)
·10
·08
·06
·04
·02
a
b
c
d
5
10
15
20
25
30
time (min)

dulcitol, no accumulation of dulcitol above the basal level was observed. In contrast to these results, strain 7009 did not accumulate dulcitol above the non-induced level after growth in RDM containing dulcitol plus 0.05% (w/v) inositol (Fig. 15b). However, when dulcitol was the sole C source present in the medium used for the culture, the bacteria of strain 7009 took up dulcitol at a rate similar to that of fully-induced bacteria of strain 7008 (Fig. 15b). In this experiment the bacteria were exposed to dulcitol for only 16 h so it is unlikely that revertants arose. However, if glucose was included in the medium, together with dulcitol, no dulcitol transport activity was induced in strain 7009 although this strain was unable to metabolize glucose as a consequence of the glk-4 mutation. Therefore, the induction of dulcitol transport activity in strain 7009 was very similar to that observed with strain 7008, except for its hypersensitivity to repression by inositol.

Strain 7010 took up dulcitol at a similar rate irrespective of whether the bacteria were grown in the presence of dulcitol plus inositol, dulcitol plus glucose or inositol alone (Fig. 15c) indicating that dulcitol uptake was constitutive in this strain. If the bacteria were grown with dulcitol as sole C source, they transported dulcitol at a 40% faster rate (Fig. 15c), indicating that growth in the presence of inositol or glucose still caused a repressive effect on the amount of dulcitol transport activity in the strain. However, the repression of dulcitol transport activity caused by the presence of glucose in the growth medium of strain 7010 was far less than that observed under identical conditions in strain 7008. Results obtained with strain 7011 (Fig. 15d) were very similar to those obtained with strain 7010.

Thus the results obtained by assaying the activity of the dulcitol transport system were very similar to those obtained by assaying dulcitol dehydrogenase. The dulcitol transport system was not induced in strain 7009 if a second carbon source was present, but

was expressed constitutively in dulcitol specific revertants of strain 7009. These results again suggested that the Glk$^-$ phenotype may be mediated by inducer exclusion, at least in part. However, the dulcitol-transport activity was fully induced in strain 7009 if the bacteria were cultured overnight with dulcitol as sole carbon source. This result, which was obtained in each of the four independent experiments, was surprising. At the time at which the bacteria were harvested, the absorbance (A_{540}) of the cultures was not more than 0.02 and the bacteria were therefore growing at the rate characteristic of the initial growth phase (cf. Fig. 5e). Nevertheless, if the bacteria were harvested when the culture had reached an absorbance of 0.10 and growth was therefore occurring at the rate characteristic of growth on dulcitol (cf. Fig. 5e), no difference in dulcitol transport activity was observed. Thus these experiments did not provide an explanation for the impaired growth of strain 7009 on dulcitol. Similarly, no explanation for the greatly improved growth rate of strain 7010 on dulcitol was obtained. The only conclusion that could be drawn was that the induction of the dulcitol transport system in strain 7009 was hypersensitive to repression by a second carbon source, as was found for dulcitol dehydrogenase in the same strain.

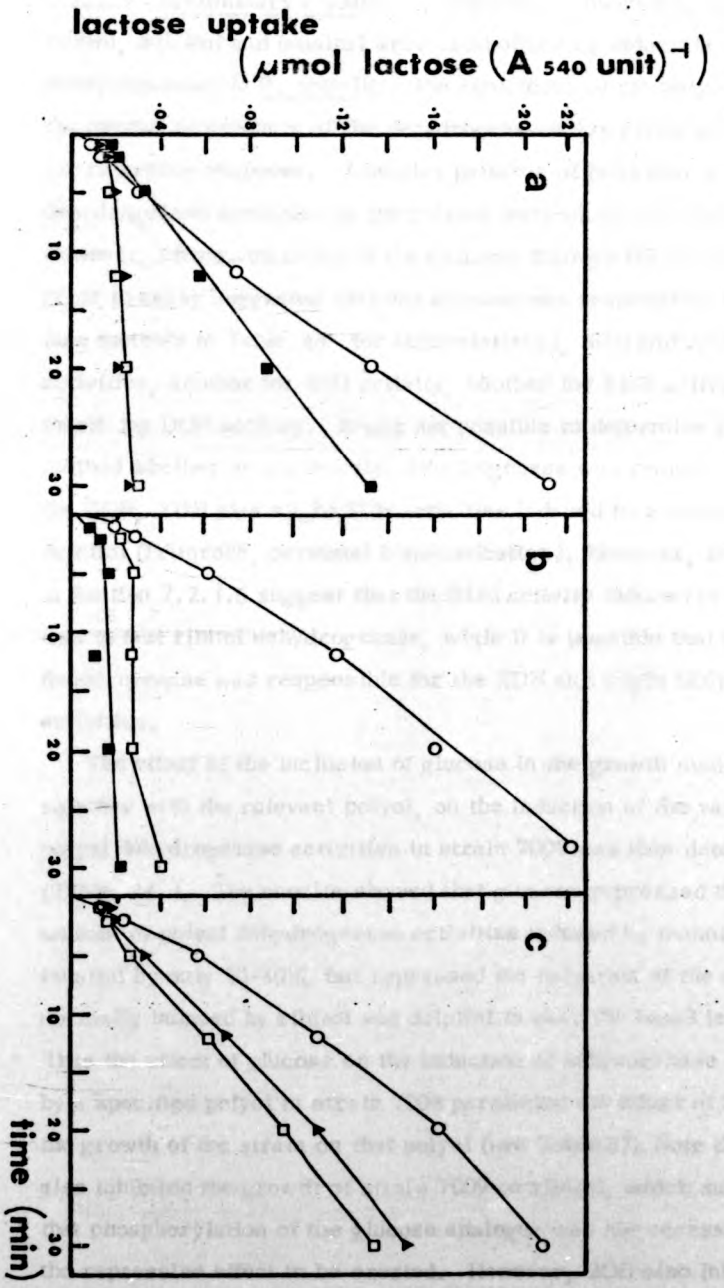
7.2.1.9 The transport of lactose by strains 7008, 7009 and 7029. - Very similar results were found for lactose transport by strains 7008, 7009 and 7029 (Fig. 16, a, b and c respectively) as were found for dulcitol transport by strains 7008, 7009 and 7010 in the previous section. Therefore synthesis of the lactose transport system was hypersensitive to repression by a second carbon source in strain 7009 and was constitutive in the lactose-specific revertants. In the wild-type strain, the transport system was not induced if glucose was present in the medium.

Fig. 16 Lactose transport by strains 7008, 7009 and 7029

See legend to Fig. 15 for growth conditions. The carbon sources used for the growth of the bacteria were:-

○, lactose; ■, lactose + inositol (0.1% w/v); □, lactose + glucose; ▲, inositol.

(a) strain 7008; (b) strain 7009; (c) strain 7029.

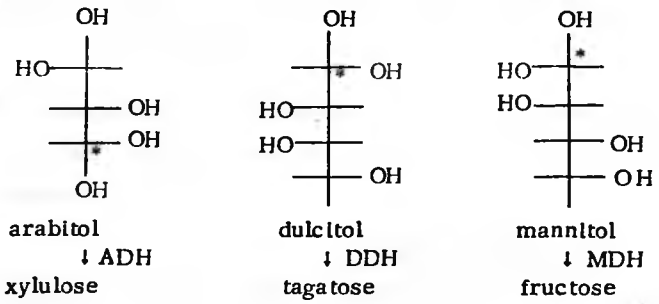

lactose uptake
(μmol lactose (A 540 unit)⁻¹)
·04
·08
·12
·16
·20
·22
a
b
c
10
20
30
time (min)

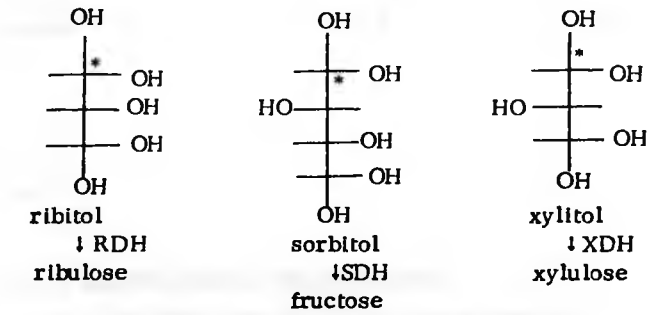
7.2.2 Studies on the effect of glucose on the metabolism of polyols by strains 7008 and 7009

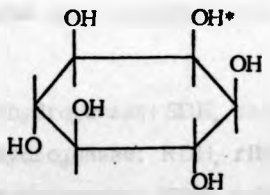
7.2.2.1 Preliminary studies.- The polyols mannitol, arabitol, ribitol, dulcitol and inositol were catabolized by inducible dehydrogenases in R. trifolii. The structures of the polyols, and the presumed products of the dehydrogenases are given in Fig. 17 for reference purposes. Complex patterns of induction of polyol dehydrogenase activities by the polyols were observed (Table 43). However, chromatography of the extracts through DEAE cellulose prior to assay suggested that one enzyme was responsible for MDH (see footnote to Table 43 for abbreviations), SDH and ADH activities, another for IDH activity, another for RDH activity and a fourth for DDH activity. It was not possible to determine by this method whether or not dulcitol dehydrogenase was responsible for the RDH, XDH plus slight SDH activities induced by growth on dulcitol (Primrose, personal communication). However, the data in Section 7.2.1.6 suggest that the RDH activity induced by dulcitol was in fact ribitol dehydrogenase, while it is possible that dulcitol dehydrogenase was responsible for the XDH and slight SDH activities.

The effect of the inclusion of glucose in the growth medium, together with the relevant polyol, on the induction of the various polyol dehydrogenase activities in strain 7008 was then determined (Table 44). The results showed that glucose repressed the amount of polyol dehydrogenase activities induced by mannitol or inositol by only 20-40%, but repressed the induction of the activities normally induced by ribitol and dulcitol to near the basal level. Thus the effect of glucose on the induction of dehyrogenase activities by a specified polyol in strain 7008 paralleled the effect of 2DG on the growth of the strain on that polyol (see Table 37). Note that 2DG also inhibited the growth of strain 7009 on ribitol, which suggested that phosphorylation of the glucose analogue was not necessary for the repressive effect to be exerted. However, 2DG also inhibited

Fig. 17 The structure of the polyols, and the presumed products to which they are metabolized by the dehydrogenase







myo-inositol
↓ IDH
2-keto-myo-inositol

* Site at which oxidation occurs

Table 43 Induction of polyol dehydrogenase activities by polyols in R. trifolii 7000*

Bacteria were grown in RDM containing the specified polyol as sole C source. Extracts were prepared and assayed as described in Chapter 2.

	Dehydrogenase** activity						
Grown on	MDH	SDH	ADH	RDH	DDH	XDH	IDH
Mannitol	+	+	+	-	-	-	-
Sorbitol	+	+	+	-	-	+	-
Arabitol	+	+	+	-	-	-	-
Ribitol	+	+	+	+	-	-	-
Dulcitol	-	±	-	+	+	+	-
Inositol	-	-	-	-	-	-	+
Xylitol	-	-	-	-	-	-	-

Key: +, dehydrogenase activity induced
±, very slight dehydrogenase activity induced
-, dehydrogenase activity not induced.

* The data in this table were compiled with the aid of Dr. S.B. Primrose.

** MDH, mannitol dehydrogenase; SDH, sorbitol dehydrogenase; ADH, arabitol dehydrogenase; RDH, ribitol dehydrogenase; DDH, dulcitol dehydrogenase; XDH, xylitol dehydrogenase; IDH, inositol dehydrogenase.

Table 44 The effect of glucose on the induction of dehydrogenase activities by various polyols in R. trifolii 7008

The conditions used were as described in the legend to Table 38, except that the bacteria used for inoculation were grown on RDM-galactose plates.

	Dehydrogenase* activity			
Carbon source(s)	MDH	IDH	RDH	DDH
Mannitol	378	-**	-	-
Mannitol + glucose	265	-	-	-
Inositol	-	756	-	-
Inositol + glucose	-	506	-	-
Ribitol	230	-	139	-
Ribitol + glucose	26	-	25	-
Dulcitol***	-	-	272	250
Dulcitol + glucose***	-	-	23	25

* Abbreviations are given in the footnote to Table 43.

** -, not present

*** from data in Table 38.

the growth on dulcitol of strain 7010, in which dulcitol dehydrogenase was constitutive and not sensitive to repression by glucose (Table 40). This suggested that the inhibition caused by 2DG was not manifested at the level of the synthesis of either the transport system or the catabolic enzyme(s). This was confirmed by the finding that strain 7011, which grew on dulcitol in the presence of 2DG, contained the same amount of the dulcitol transport system and dulcitol dehydrogenase as strain 7010 (Sections 7.2.1.6 and 7.2.1.8).

A further paradox was the fact that strain 7009 grew on ribitol in the presence of glucose at a rate similar to its growth on ribitol alone. This suggested that ribitol dehydrogenase was not repressed by glucose in this strain, and thus that phosphorylation of glucose was required if it was to repress the synthesis of ribitol dehydrogenase. Therefore, it was decided to compare the effect of glucose on the induction of the ribitol transport system and of ribitol dehydrogenase in strains 7008 and 7009. For comparative purposes, similar experiments were done using mannitol in the place of ribitol.

7.2.2.2 The effect of glucose and of inositol on the induction of ribitol dehydrogenase and the ribitol transport system in strains 7008 and 7009.- In order to yield directly comparable results, the activities of ribitol dehydrogenase and of the ribitol transport system were assayed in bacteria prepared from the same culture. The activities found for ribitol dehydrogenase are shown in Fig. 18a and those for ribitol transport are shown in Fig. 18b. It is apparent from the Figure that the ribitol transport system and ribitol dehydrogenase were co-ordinately controlled in both strains 7008 and 7009. Therefore in strain 7008 neither was induced if either glucose or inositol was present in the inducing medium. In contrast to this, in strain 7009 both activities were fully-induced when the bacteria were grown in the presence of glucose plus ribitol. However, they were repressed to the same extent as in strain 7008 if inositol was the second carbon source present.

Fig. 18 The effect of glucose and of inositol on the induction of ribitol dehydrogenase and the ribitol transport system in strains 7008 and 7009.

Bacteria were grown in 1 litre Erlenmeyer flasks containing 500 ml RDM plus the specified carbon source(s), each at 0.4% (w/v). The flasks were inoculated with bacteria suspended off RDM-galactose plates. 50 ml of culture from each flask was used for the uptake experiments and the remaining 450 ml to prepare cell-free extracts for the assay of ribitol dehydrogenase. The carbon source(s) used for the growth of the bacteria were:

○, ● , ribitol;

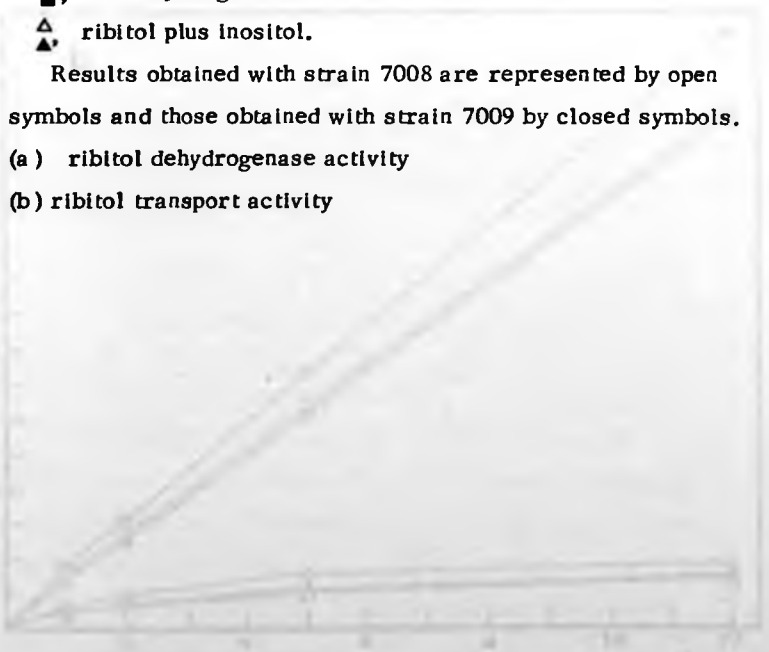
□, ■, ribitol plus glucose;

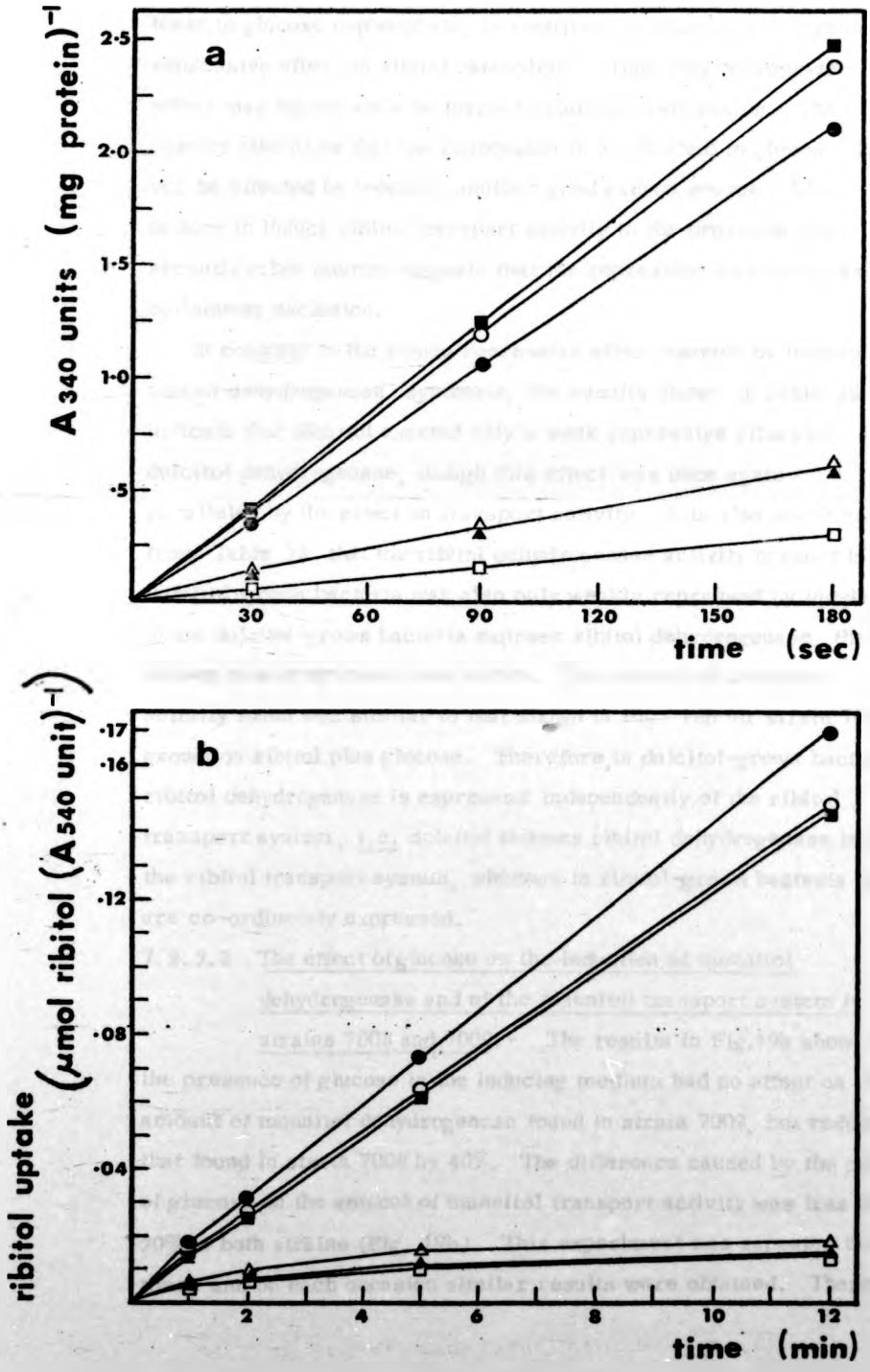
△, ▲, ribitol plus inositol.

Results obtained with strain 7008 are represented by open symbols and those obtained with strain 7009 by closed symbols.

(a) ribitol dehydrogenase activity

(b) ribitol transport activity




a
A 340 units (mg protein)$^{-1}$
2·5
2·0
1·5
1·0
·5
30
60
90
120
150
180
time (sec)
b
ribitol uptake (μmol ribitol (A 540 unit)$^{-1}$)
·17
·16
·12
·08
·04
2
4
6
8
10
12
time (min)

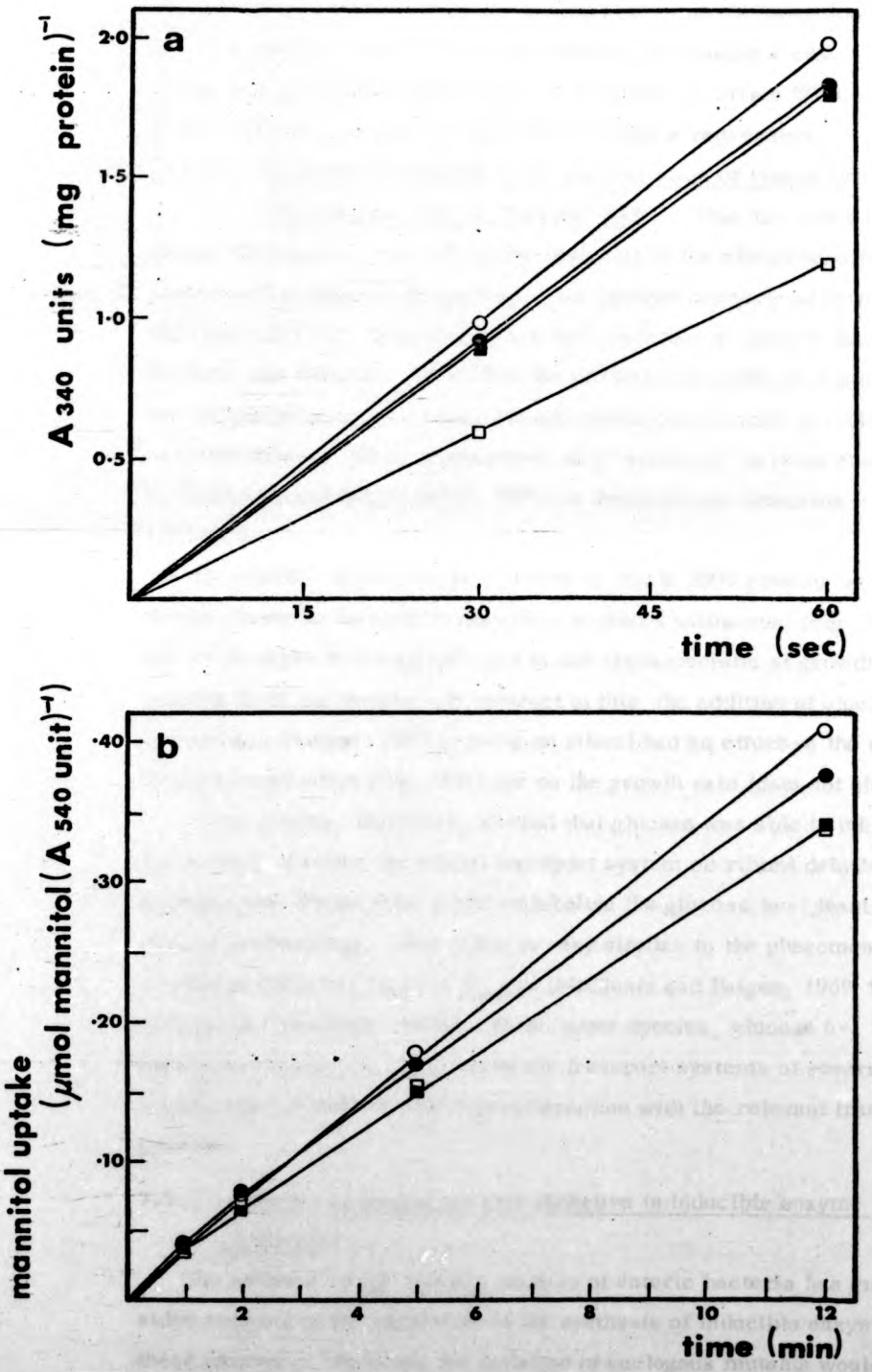
The above results indicate that the catabolism of glucose, at least to glucose 6-phosphate, is required for glucose to exert its repressive effect on ribitol catabolism. Thus this repressive effect may legitimately be termed catabolite repression. The results also show that the repression is not limited to glucose, but can be effected by inositol, another good carbon source. The failure to induce ribitol transport activity in the presence of a second carbon source suggests that the repression may be mediated by inducer exclusion.

In contrast to the strong repressive effect exerted by inositol on ribitol dehydrogenase synthesis, the results shown in Table 38 indicate that inositol exerted only a weak repressive effect on dulcitol dehydrogenase, though this effect was once again paralleled by the effect on transport activity. It is also worth noting from Table 38 that the ribitol dehydrogenase activity present in dulcitol-grown bacteria was also only weakly repressed by inositol. Since dulcitol-grown bacteria express ribitol dehydrogenase, their ability to take up ribitol was tested. The amount of transport activity found was similar to that shown in Fig. 18b for strain 7008 grown on ribitol plus glucose. Therefore, in dulcitol-grown bacteria, ribitol dehydrogenase is expressed independently of the ribitol transport system, i.e. dulcitol induces ribitol dehydrogenase but not the ribitol transport system, whereas in ribitol-grown bacteria they are co-ordinately expressed.

7.2.2.3 The effect of glucose on the induction of mannitol dehydrogenase and of the mannitol transport system in strains 7008 and 7009.- The results in Fig.19a show that the presence of glucose in the inducing medium had no effect on the amount of mannitol dehydrogenase found in strain 7009, but reduced that found in strain 7008 by 40%. The difference caused by the presence of glucose on the amount of mannitol transport activity was less than 20% in both strains (Fig. 19b). This experiment was repeated three times and on each occasion similar results were obtained. Therefore

Fig. 19 The effect of glucose on the induction of mannitol dehydrogenase and the mannitol transport system in strains 7008 and 7009

The experiments were done as described in the legend to Fig. 18. The carbon source(s) used for the growth of the bacteria were:
○, ●, mannitol; □, ■, mannitol plus glucose.
Results obtained with strain 7008 are represented by open symbols and those obtained with strain 7009 by closed symbols.
(a) mannitol dehydrogenase activity; (b) mannitol transport activity.


a
2·0
1·5
1·0
0·5
A 340 units (mg protein)⁻¹
15
30
45
60
time (sec)
b
·40
·30
·20
·10
mannitol uptake
(μmol mannitol (A 540 unit)⁻¹)
2
4
6
8
10
12
time (min)

the presence of glucose caused a minimal effect on the induction of mannitol transport activity in either strain, but caused a weak repression of mannitol dehydrogenase synthesis in strain 7008. Strain 7009 was resistant to this weak catabolite repression.

7.2.2.4 The effect of glucose on the incorporation of ribitol by ribitol-grown strains 7008 and 7009. - Thus far it has been shown that glucose could inhibit the induction of the ribitol transport system and ribitol dehydrogenase in non-induced bacteria of strain 7008 but not 7009. Whether glucose had any effect on fully-induced bacteria was unknown. Therefore the effect of the addition of glucose to cultures growing on ribitol upon the continued utilization of ribitol was determined. These experiments were analogous to those done by McGinnis and Paigen (1969, 1973) to demonstrate catabolite inhibition.

The addition of glucose to a culture of strain 7008 growing on ribitol caused an immediate cessation of ribitol utilization (Fig. 20a) and an increase in the growth rate to one characteristic of growth on glucose (data not shown). In contrast to this, the addition of glucose to a culture of strain 7009 growing on ribitol had no effect on the rate of ribitol utilization (Fig. 20b) nor on the growth rate (data not shown).

These results, therefore, showed that glucose was able to inhibit the activity of either the ribitol transport system or ribitol dehydrogenase, provided that the bacteria could metabolize the glucose to at least glucose 6-phosphate. This effect is very similar to the phenomenon of catabolite inhibition found in E. coli (McGinnis and Paigen, 1969, 1973; Amaral and Kornberg, 1975). In the latter species, glucose 6-phosphate inhibits the activities of the transport systems of several sugars, possibly by an allosteric interaction with the relevant transport protein.

7.2.3 Attempts to isolate mutants defective in inducible enzyme synthesis

The isolation of cya and crp mutants of enteric bacteria has greatly aided analysis of the regulation of the synthesis of inducible enzymes in those species. Obviously the isolation of analogous mutants would be

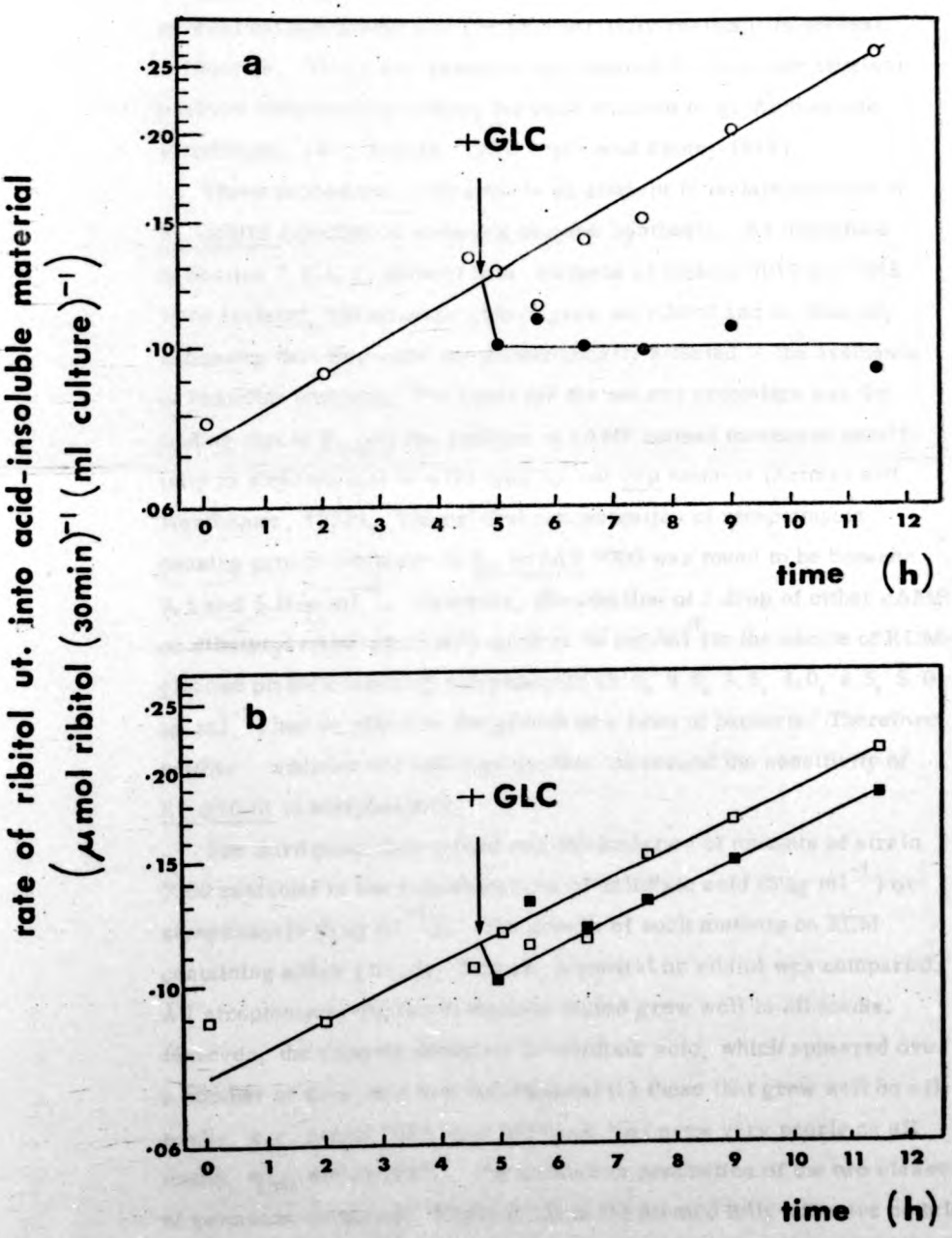
Fig. 20 The effect of glucose on the utilization of ribitol by strains 7008 and 7009

The method used for this experiment was based on that of McGinnis and Paigen (1969). Overnight cultures growing exponentially in RDM containing ribitol (0.4% w/v) as sole C source were diluted to give approximately 10^8 bacteria ml^{-1} in 250 ml of pre-warmed RDM-ribitol medium contained in 500 ml Erlenmeyer flasks (t = 0 h). At the point indicated in the Figure (t = 4.75 h), each culture was divided equally between two sterile pre-warmed 250 ml Erlenmeyer flasks. Glucose (0.4% w/v) was added to one flask (closed symbol) and the other was used as a control (open symbol).

Three-ml samples were taken from the various flasks at the selected times, and 1 ml was used for determining the absorbance (A_{540}) of the culture whereas the other 2.0 ml were transferred to a 25 ml Erlenmeyer flask containing 0.4 µCi of ^{14}C-labelled ribitol (25 µCi ml^{-1}: 1.88 µCi µmol^{-1}). After incubation of the sample flasks with gentle shaking in a water-bath (28° C) for 30 min, a 1.0 ml sample was removed from each flask and placed into a test-tube containing 0.1 ml of 4% formaldehyde. The tubes were then heated at 100° C for 5 min, chilled and the acid-insoluble material precipitated by the addition of 2.0 ml of cold 0.3 M trichloroacetic acid. The samples were then filtered on to Millipore discs (0.45 µm pore size; 25 mm diameter) and were given four washes with 2.0 ml volumes of 0.3 M trichloroacetic acid, followed by two washes with 5.0 ml volumes of distilled water. The filters were then dried and counted.

(a) Strain 7008

(b) Strain 7009


a
+GLC
·25
·20
·15
·10
·06
0 1 2 3 4 5 6 7 8 9 10 11 12
time (h)
b
+GLC
·25
·20
·15
·10
·06
0 1 2 3 4 5 6 7 8 9 10 11 12
time (h)
rate of ribitol ut. into acid-insoluble material
(μmol ribitol (30min)⁻¹ (ml culture)⁻¹)

useful for the study of the regulation of metabolism in R. trifolii. The cya and crp mutants of enteric bacteria are unable to grow on several carbohydrates and are also partially resistant to several antibiotics. This latter property has formed the basis for efficient positive selection procedures for such mutants (e.g. Artman and Werthamer, 1974; Kumar, 1976; Alper and Ames, 1978).

Three procedures were used in an attempt to isolate mutants of R. trifolii defective in inducible enzyme synthesis. As described in Section 7.2.1.2, several Sam$^-$ mutants of strains 7012 and 7013 were isolated, but all were able to grow on ribitol and on inositol, indicating that they were not pleiotropically affected in the synthesis of inducible enzymes. The basis for the second procedure was the finding that in E. coli the addition of cAMP caused increased sensitivity to streptomycin in wild-type but not crp mutants (Artman and Werthamer, 1974). The critical concentration of streptomycin causing growth inhibition in R. trifolii 7000 was found to be between 2.5 and 5.0 $\mu g\ ml^{-1}$. However, the addition of 1 drop of either cAMP or dibutyryl cAMP (dbcAMP) (each at 16 mg/ml^{-1}) to the centre of RDM-glucose plates containing streptomycin (2.5, 3.0, 3.5, 4.0, 4.5, 5.0 $\mu g\ ml^{-1}$) had no effect on the growth of a lawn of bacteria. Therefore neither addition of cAMP nor dbcAMP increased the sensitivity of R. trifolii to streptomycin.

The third procedure tested was the isolation of mutants of strain 7000 resistant to low concentrations of nalidixic acid (5 $\mu g\ ml^{-1}$) or streptomycin (5 $\mu g\ ml^{-1}$). The growth of such mutants on RDM containing either glucose, lactose, mannitol or ribitol was compared. All streptomycin-resistant mutants tested grew well in all media. However, the mutants resistant to nalidixic acid, which appeared over a number of days, fell into two classes: (i) those that grew well on all media, e.g. strain 7084, and (ii) those that grew very poorly on all media, e.g. strain 7085. The nodulation properties of the two classes of mutant were tested. Those in class (i) formed fully-effective nodules whereas those in class (ii) formed normal sized nocules but the nodules were pure white and did not reduce even a trace of acetylene.

The growth of strain 7085 on a number of carbohydrates was tested but it grew equally poorly on all. Also the addition of cAMP, dbcAMP or cGMP had no effect on the growth rate of the strain, suggesting that the strain was not defective in cyclic nucleotide metabolism although it is unknown whether the nucleotides were taken up by the bacteria. However, an interesting analogy to ATPase-deficient mutants of Bacillus megaterium (Decker and Lang, 1977) is suggested. These mutants which were isolated as neomycin-resistant were unable to grow on nonfermentable carbon sources. If the slow-growing nalidixic acid-resistant mutants of R. trifolii were ATPase-deficient, this would explain their ineffectiveness since ATPase has a role in maintaining the energized state of the cell membrane (see Decker and Lang, 1977). This state is essential for nitrogen fixation in bacteroids (Laane et al., 1978). Unfortunately the failure to find a medium on which strain 7084 would grow well precluded a more thorough examination of it, but this is an area where further study may prove rewarding.

7.3 Discussion

The results reported in this chapter raise more problems than they resolve. The work done was aimed at providing information on the regulation of carbohydrate metabolism in R. trifolii and as such can be divided into two sections: (i) studies on the causation of the Glk^- phenotype and (ii) studies on the sensitivity of inducible enzyme synthesis to catabolite repression.

The isolation of only the two classes of revertants (wild-type and 'sugar-specific') from the glk mutants suggested that a secondary messenger such as cAMP or an RPr-type protein was not involved in the causation of the Glk^- phenotype. If a secondary messenger was involved, the isolation of revertants able to grow on sucrose, lactose and dulcitol, but not glucose, would have been expected. The fact that all glucose-positive revertants displayed a wild-type phenotype, but not all regained glucokinase, suggested that glucokinase per se was not

involved in the causation of the Glk$^-$ phenotype, but more probably the phenotype resulted from the inability to metabolize glucose. The causation of the defective growth on dulcitol and on lactose were then investigated further as model systems. The former was chosen because its catabolism presumably does not generate glucose whereas the latter was chosen because its catabolism does generate glucose.

A defect in the induction of dulcitol dehydrogenase as a causal agent for the defective growth of strain 7009 on dulcitol was indicated by two findings. Firstly, it was found that in strain 7009 dulcitol dehydrogenase was not synthesized if a second carbon source was present in the growth medium along with dulcitol. Secondly, it was found that the dulcitol-specific revertants of strain 7009 (e.g. strain 7010) synthesized dulcitol dehydrogenase constitutively. However, paradoxically it was then found that dulcitol dehydrogenase was synthesized in strain 7009 if a second carbon source was not present. A second paradoxical finding was that although strain 7010 grew much faster on dulcitol than strains 7008 and 7009, it contained enzyme activities and amounts identical to those in strains 7008 and 7009 grown on dulcitol. The possibility that differences in the kinetics of induction of dulcitol dehydrogenase in strains 7008 and 7009 were responsible for the difference in the growth of the two strains on dulcitol seems unlikely since the growth-rate of the strains on dulcitol was constant and characteristic of that strain (Fig. 5e). Therefore, studies on the induction of dulcitol dehydrogenase in strain 7009 indicated that it was hypersensitive to repression by a second carbon source, and constitutive in dulcitol-specific revertants of that strain. The latter point suggested very strongly that the Glk$^-$ phenotype was manifested at the level of inducer exclusion, at least in part. Since β-galactosidase was a constitutive enzyme in strain 7008, similar studies on the presumed first enzyme of lactose catabolism were inconsequential. Certainly no differences in β-galactosidase levels in strains 7008, 7009 or 7029 were detected.

Results from studies on the induction of the dulcitol and the lactose transport systems closely paralleled those obtained from the study of the induction of dulcitol dehydrogenase. Therefore, in both cases the respective transport system was not induced in strain 7009 if it was cultured in the presence of a second carbon source, but was induced if the respective inducing sugar was the sole carbon source present. The respective transport systems were expressed constitutively in the respective sugar-specific revertants. It was possible to test the effect of glucose on the induction of the transport systems in strain 7009, in addition to the other strains, since cultures at low cell-densities could be used to prepare bacteria for the uptake experiments. This overcame the difficulties encountered when attempting to do similar experiments on the induction of dulcitol dehydrogenase. It was found that glucose repressed the synthesis of both transport systems in strain 7009 as well as in strain 7008 (Figs. 15a, b and 16a, b), but only had a slight effect on the amount of transport activity in the constitutive revertant strains (Figs. 15c and 16c).

Therefore, taken overall, studies on the amounts of the lactose and dulcitol transport systems did not provide a ready explanation for the Glk$^-$ phenotype. They also did not provide an explanation for the faster growth of strains 7010 and 7011 on dulcitol. However, the finding that the sugar-specific revertants synthesized the transport systems constitutively once again strongly suggested that inducer exclusion was involved in the causation of the Glk$^-$ phenotype. Nevertheless, the paradox implied by the finding of wild-type amounts of transport activity in strain 7009 cultured in the presence of the inducing sugar only could not be resolved.

It should be noted that no studies on the mechanism of dulcitol or lactose transport were done. However, both sugars were taken up much more slowly than the other sugars tested (glucose, fructose, mannitol, ribitol) by induced bacteria of strain 7008, suggesting that the basic mechanism used for the transport of dulcitol and lactose was different from that used for the transport of the other sugars.

Therefore it is possible that the causation of the Glk$^-$ phenotype may somehow be tied in with the mechanism by which the bacteria take up the affected sugars.

Thus the results obtained from the various experiments require that any hypothesis for the causation of the Glk$^-$ phenotype takes into account the following observations:-

(i) the phenotype is caused by the inability to metabolize glucose;

(ii) a secondary messenger is not involved:

(iii) the amounts of the relevant transport systems and catabolic enzymes in the Glk$^-$ strain are similar to those in the wild-type strain:

(iv) the growth rate of the Glk$^-$ strain on a particular affected sugar is constant and characteristic for that sugar;

(v) the synthesis of the relevant transport system and, in the case of dulcitol, catabolic enzyme is hypersensitive to repression by a second carbon source, including glucose, in the Glk$^-$ strain;

(vi) sugar-specific revertants synthesize the relevant transport system, and, in the case of dulcitol, catabolic enzyme constitutively;

(vii) the sugar-specific revertants grow faster on the specific sugar than the wild-type bacteria do. Note that this effect was especially pronounced for the dulcitol-specific revertant but was also found for the lactose-specific revertant;

(viii) the growth of the sugar-specific revertants on the specific sugar was inhibited by 2DG to a similar extent as the growth of the wild-type bacteria.

The major difficulty in developing a hypothesis to account for the Glk$^-$ phenotype was to reconcile observations (iii) and (vi, above into a unifying concept. However, a model which accounts for most of the observations was evolved. It is proposed that in non-induced cells the affected sugar enters the bacteria by a low affinity transport protein, TPr, and induces the synthesis of the sugar-specific transport protein SPr. SPr then inserts into the membrane in such a way that

TPr is masked. However, during the initial entry of the sugar, its interaction with TPr results in the synthesis or activation of a protein CPr. CPr is able to weakly bind glucose, and when glucose is bound to it, the resultant protein CPr-Glc is able to interact with SPr and inhibit its activity.

The model accounts for the observations listed above in order as follows:-

(i) metabolism of glucose keeps the level of glucose in the cell low enough so that most CPr is in the free form;

(ii) the model contradicts this observation, since CPr is postulated as a secondary messenger, the loss of which by mutation would relieve the block in dulcitol and lactose metabolism. It is, of course, possible that such a mutation could have occurred, but its frequency would have had to be very low since the Dul^+ Lac^+ Glc^- phenotype was not detected. A second possibility would be that the strains contained more than one gene specifying CPr, or more than one protein with CPr activity, in which case mutants devoid of CPr would not be detected:

(iii) the normal transport of dulcitol and lactose would be explained if the washing of the cells prior to doing the transport experiments reduced the concentration of glucose in the cells so that CPr was in the free form. Note that CPr would be in the glucose-bound form in the Glk^- strains, since glucose would be generated internally from most of the affected sugars. However, glucose would presumably have to be formed via glucose 6-phosphatase from glucose 6-phosphate generated from dulcitol and lactulose in the case of the two sugars. Note that the severity of the Glk^- effect on those two sugars was less than on sucrose or lactose;

(iv) the growth rate of the bacteria would be limited by the rate at which the SPr-CPr-Glc complex transported the sugars:

(v) catabolite repression, in enteric bacteria at least, is controlled by both inducer level and cAMP levels. The model proposes that the level of inducer in the Glk^- cells is very low, so therefore only small

TPr is masked. However, during the initial entry of the sugar, its interaction with TPr results in the synthesis or activation of a protein CPr. CPr is able to weakly bind glucose, and when glucose is bound to it, the resultant protein CPr-Glc is able to interact with SPr and inhibit its activity.

The model accounts for the observations listed above in order as follows:-

(i) metabolism of glucose keeps the level of glucose in the cell low enough so that most CPr is in the free form;

(ii) the model contradicts this observation, since CPr is postulated as a secondary messenger, the loss of which by mutation would relieve the block in dulcitol and lactose metabolism. It is, of course, possible that such a mutation could have occurred, but its frequency would have had to be very low since the Dul^+ Lac^+ Glc^- phenotype was not detected. A second possibility would be that the strains contained more than one gene specifying CPr, or more than one protein with CPr activity, in which case mutants devoid of CPr would not be detected:

(iii) the normal transport of dulcitol and lactose would be explained if the washing of the cells prior to doing the transport experiments reduced the concentration of glucose in the cells so that CPr was in the free form. Note that CPr would be in the glucose-bound form in the Glk^- strains, since glucose would be generated internally from most of the affected sugars. However, glucose would presumably have to be formed <u>via</u> glucose 6-phosphatase from glucose 6-phosphate generated from dulcitol and lactulose in the case of the two sugars. Note that the severity of the Glk^- effect on those two sugars was less than on sucrose or lactose;

(iv) the growth rate of the bacteria would be limited by the rate at which the SPr-CPr-Glc complex transported the sugars:

(v) catabolite repression, in enteric bacteria at least, is controlled by both inducer level and cAMP levels. The model proposes that the level of inducer in the Glk^- cells is very low, so therefore only small

changes in the level of the secondary effector of catabolite repression (cAMP ?) would be required to exert catabolite repression. The effect of glucose on the transport activity in the Glk$^-$ strains may be accounted for if it increases the intracellular level of glucose, such that CPr is in the bound form even after the washing of the bacteria. The extracellular glucose might induce a system capable of concentrating glucose to a greater degree than found in non-induced bacteria, i.e. the level of glucose would be higher than ever attained by intracellularly-generated glucose:

(vi) bacteria in which SPr was synthesized constitutively would not take up the sugar by TPr; therefore CPr would not be induced;

(vii) in wild-type bacteria, some CPr would be in the form CPr-Glc, and therefore a certain proportion of SPr would always be in the inactive form SPr-CPr-Glc. Thus, in wild-type bacteria, the growth rate would be limited by the amount of free SPr. Sugar-specific revertants which do not synthesize CPr would take up the sugar at maximal rates, explaining their faster growth rate;

(viii) to account for the inhibition of growth by 2DG, an allosteric binding site on SPr sensitive to 2DG has to be invoked.

The model is obviously highly speculatory and less than satisfactory in several respects. The major weaknesses are in the explanations of observations (ii) and (iii) above. However, a more simple model to account for the experimental findings could not be devised.

As noted above, there was a disparity between the effect of 2DG and of glucose on the growth of strain 7010 on dulcitol and strain 7029 on lactose. This disparity was also observed for the growth of strains 7009 and 7010 on sugars whose metabolism was not affected by the glk mutation (i.e. ribitol and xylitol). It was found that 2DG inhibited the growth of strains 7008, 7009 and 7010 on those sugars whose utilization was subject to catabolite repression by glucose in strain 7008. It is proposed that the permeases for the sugars that are subject to catabolite repression possess an allosteric regulatory site which binds to glucose 6-phosphate and 2DG with high affinity and to

glucose with very low affinity, i.e. 2DG acts as a metabolic analogue of glucose 6-phosphate rather than glucose. This would explain the differential effect of 2DG and glucose on the growth of strains 7009 and 7010. Certainly the finding that 2DG inhibited the growth of strain 7010 on dulcitol is indicative of another form of control of carbohydrate utilization. Strain 7011, which was the only 2DG-resistant isolate found, grew on dulcitol in the presence of 2DG, but 2DG still inhibited its growth on ribitol, lactose, etc. (see Table 37), i.e. only the utilization of dulcitol was resistant to 2DG. The mutation(s) that gave rise to strain 7011 was associated with a number of other phenotypic effects, but it is obvious that genetic analysis of the strain is required before their significance can be assessed. Note that no mutants able to grow on dulcitol plus 2DG were isolated from strain 7008, although an extensive search for them was made. This provides further suggestive evidence for the dual control of dulcitol metabolism by glucose, i.e. the Glk$^-$ effect mediated by CPr-glucose (or CPr-2DG) and the allosteric regulation of the transport protein. This dual mode of control suggests a reason why only dulcitol specific revertants were isolated from strain 7009 cultured on media containing dulcitol plus 2DG. Since the revertants were sensitive to only one mode of control, they were able to grow slowly on the medium, whereas glucose-positive revertants, sensitive to both modes of control, were doubly inhibited.

In addition to the above studies on the causation of the Glk$^-$ phenotype, the control of the synthesis of inducible enzymes required for the catabolism of polyols was studied in strains 7008 and 7009. As already noted, the effect of 2DG on the growth of strain 7008 on a particular polyol was similar to the effect of glucose on the induction of the relevant polyol dehydrogenase. Therefore, the syntheses of SAM dehydrogenase and inositol dehydrogenase were relatively unaffected by the presence of glucose, whereas the syntheses of ribitol dehydrogenase and dulcitol dehydrogenase were repressed by the presence of glucose. However, in strain 7009 the synthesis of ribitol dehydrogenase was not repressed by the

presence of glucose, showing that the metabolism of glucose to at least glucose 6-phosphate was required for the repression to be exerted. In both strains 7008 and 7009, the synthesis of ribitol dehydrogenase was repressed by the presence of inositol in the medium. In most cases, the activity of the respective transport systems paralleled the activity of the dehydrogenase. A possible exception occurred with mannitol where the amount of transport system synthesized was relatively unaffected by glucose, but the amount of mannitol dehydrogenase was repressed 40%. This repression was also dependent on the metabolism of glucose to at least glucose 6-phosphate, since it did not occur in strain 7009.

The catabolite repression of polyol dehydrogenases in R. trifolii seems to follow the same basic principle as catabolite repression in other genera: the presence of a good carbon source represses the formation of inducible enzymes required to catabolize poor carbon sources (e.g. ribitol, dulcitol, lactose), but not the formation of inducible enzymes required to catabolize other good carbon sources (e.g. inositol, mannitol). It is perhaps not surprising that R. trifolii does not regulate the synthesis of all inducible enzymes in response to glucose in the medium, since in its natural habitat, the soil, the supply of carbohydrate is likely to be a major factor limiting growth. Since the bacteria can accumulate vast quantities of poly-β-hydroxybutyrate (up to 50% of cellular dry-weight), it would seem advantageous for them to assimilate as much available carbohydrate as possible, rather than to regulate carbon metabolism to their immediate needs.

The experiments showing that the ribitol uptake system was not induced in bacteria cultured in the presence of glucose suggested that the catabolite repression may act by inducer exclusion. However, all experiments thus far described have examined the effect of glucose on the induction of the synthesis of the particular enzymes. Experiments with strain 7010 showed that the constitutive dulcitol-catabolizing components were relatively insensitive to catabolite

repression, providing further evidence that the catabolite repression may operate by inducer exclusion, at least in part. However, the dulcitol system was subject to the Glk⁻ effect and may not have been representative. Therefore the effect of glucose on the activity of the components of the ribitol catabolic system was examined (Fig. 20). The addition of glucose to a culture of strain 7008 growing on ribitol caused an immediate inhibition of ribitol utilization. This inhibition was dependent upon the catabolism of glucose since the addition of glucose to a similar culture of strain 7009 had no effect on the continued utilization of ribitol. This was, of course, expected since strain 7009 grew normally on glucose plus ribitol. All experiments indicated that ribitol catabolism was not affected by glucose unless the glucose was catabolized to at least glucose 6-phosphate. The finding that strain 7009 would not grow on ribitol plus 2DG provided the evidence that 2DG was in fact acting as a metabolic analogue of glucose 6-phosphate rather than of glucose. As already suggested, this would be explained if 2DG had a high affinity for the allosteric regulatory site on the affected permeases. Another possibility could be that 2DG was concentrated to a higher level than glucose in the glk bacteria. This would explain the effect, provided that the regulatory site in the permease had a weak affinity for glucose.

The findings suggest that catabolite repression in R. trifolii is largely mediated through inducer exclusion. A number of other findings supported this suggestion. For examples, in strain 7010 the constitutive dulcitol dehyrogenase and dulcitol transport system were relatively insensitive to catabolite repression by fructose. Also, in strain 7008 the ribitol dehydrogenase induced by dulcitol was relatively insensitive to repression by inositol as was dulcitol dehydrogenase, but the ribitol dehydrogenase induced by ribitol was sensitive to repression by inositol. Since the two ribitol dehydrogenases were probably the same enzyme, this suggests that the amount of enzyme synthesized depended upon the amount of inducer present in the cell. Similar results were found using strain 7008 with SAM dehydrogenase which was only weakly repressed by glucose when induced by mannitol, but was strongly repressed by glucose when induced by ribitol.

A basic finding was that ribitol utilization was insensitive to catabolite repression and catabolite inhibition by glucose in the mutant strain lacking glucokinase. A similar situation has been reported to occur in yeast, where mutants lacking hexokinase are insensitive to catabolite repression or inactivation (Entian, 1977; Zimmermann and Scheel, 1977). However, in yeast it is thought that hexokinase itself is a positive allosteric regulator required for catabolite inactivation (Entian, 1977), whereas its role in catabolite repression has not been defined (Zimmermann and Scheel, 1977). It is also interesting that in yeast, mutants resistant to 2DG have lost glucokinase activity (Lobo and Maitra, 1977), suggesting that in this species glucokinase can phosphorylate 2DG. However, in Pseudomonas aeruginosa 2DG is not phosphorylated by glucokinase (Mukkada et al., 1973). Which situation applies in R. trifolii remains to be determined.

7.4 Conclusions

The studies reported in this chapter are the first on the regulation of carbohydrate catabolism in Rhizobium. Evidence indicating at least three types of regulation was found. The first type of regulation was indicated by the finding that glucokinase-negative mutants were defective in growth on certain sugars apart from glucose viz. dulcitol, lactose, lactulose, raffinose and sucrose (Glk^- phenotype). The causes of the inability to grow on dulcitol and on lactose were studied in detail. Important observations were (i) the amounts of the

relevant transport systems and catabolic enzymes in the Glk$^-$ strain were similar to those in the wild-type strain; (ii) the synthesis of the relevant transport system and, in the case of dulcitol, catabolic enzyme was hypersensitive to repression by a second carbon source, including glucose, in the Glk$^-$ strain; (iii) sugar specific-revertants of the Glk$^-$ strain synthesized the relevant transport system and, in the case of dulcitol, catabolic enzyme constitutively. The major difficulty in developing a hypothesis to account for the Glk$^-$ phenotype was to reconcile observations (i) and (iii) above into a unifying concept. A model which proposes that the interaction of glucose with a regulatory protein modulates the activity of certain permeases (viz. those responsible for the transport of lactose and dulcitol) was evolved. It is obviously highly speculatory and very complex, but a more simple model to account for the experimental findings could not be devised. The features of the model (Fig. 21) are that the affected sugar enters the non-induced bacteria by a low affinity transport protein, TPr, and induces the synthesis of the sugar-specific transport protein SPr and also the activation (or synthesis) of a further protein CPr, (Fig. 21b), which can weakly bind glucose. SPr inserts into the membrane in such a way that TPr is masked, and it is then used for the transport of the sugar (Fig. 21c). However, if present, glucose binds to CPr and the resultant protein CPr-Glc is able to interact with SPr and inhibit its activity (Fig. 21d). The manner

Fig. 21. Diagrammatic representation of the model proposed to account for the inability of the Glk$^-$ strains to grow on dulcitol or lactose.

Key:


, TPr (low affinity non-specific transport protein)


, CPr (regulatory protein)


, SPr (sugar-specific transport protein)


, glucose-transport protein

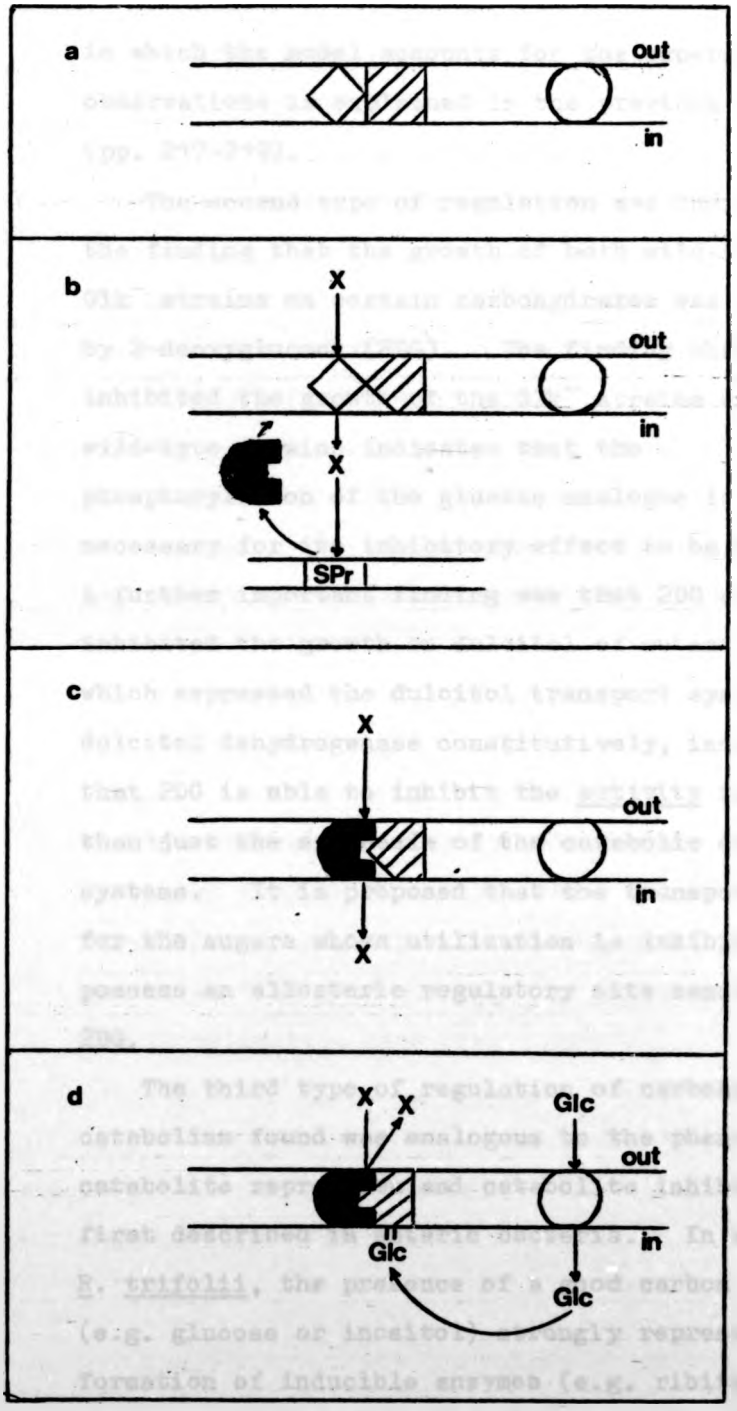
X , affected sugar

a) Non-induced cells in the absence of the affected sugar. TPr, CPr and the glucose-transport protein are constitutively expressed. CPr is present in an inactive conformational form.

b) Non-induced cells in the presence of the affected sugar. When the cells are exposed to the affected sugar, they transport it by TPr and it then induces the synthesis of SPr. During the transport of the sugar by TPr, a conformational change is induced in CPr which is then able to bind glucose.

c) Induced cells cultured in the absence of glucose. SPr has inserted into the membrane and masked TPr. The affected sugar is then transported by SPr.

d) Induced cells cultured in the presence of glucose. The interaction of intracellular glucose with CPr induces a further conformational change in CPr. CPr is then able to interact with SPr and inhibit its transport activity.


a
out
in
b
X
out
in
X
SPr
c
X
out
in
X
d
X
X
Glc
out
in
Glc
Glc

in which the model accounts for the experimental observations is explained in the previous section (pp. 217-219).

The second type of regulation was indicated by the finding that the growth of both wild-type and Glk$^-$ strains on certain carbohydrates was inhibited by 2-deoxyglucose (2DG). The finding that 2DG inhibited the growth of the Glk$^-$ strains as well as wild-type strains indicates that the phosphorylation of the glucose analogue is not necessary for its inhibitory effect to be exerted. A further important finding was that 2DG also inhibited the growth on dulcitol of mutant strains which expressed the dulcitol transport system and dulcitol dehydrogenase constitutively, indicating that 2DG is able to inhibit the activity rather than just the synthesis of the catabolic enzyme systems. It is proposed that the transport proteins for the sugars whose utilization is inhibited by 2DG possess an allosteric regulatory site sensitive to 2DG.

The third type of regulation of carbohydrate catabolism found was analogous to the phenomena of catabolite repression and catabolite inhibition first described in enteric bacteria. In wild-type R. trifolii, the presence of a good carbon source (e.g. glucose or inositol) strongly represses the formation of inducible enzymes (e.g. ribitol dehydrogenase, dulcitol dehydrogenase) required to catabolize poor carbon sources (ribitol, dulcitol) but not the formation of inducible enzymes required

CHAPTER 8

FINAL COMMENTS

Much progress has been made in this study, especially considering the work was done from scratch rather than as part of an established research project. Perhaps the most satisfying aspect of this study was the number of new research lines which were suggested by the results gained. These have been indicated in the relevant sections.

The work on carbohydrate catabolism and its control in R. trifolii (Chapters 6 and 7) has significantly advanced knowledge of a hitherto neglected but nevertheless very important aspect of Rhizobium physiology. Perhaps the most important finding was that hexoses were not the major energy-source received by the bacteroids from the nodule cytosol. The isolation of carbohydrate-defective mutants of free-living rhizobia was probably the most powerful technique that could be applied to the study of this problem. The difficulties found in biochemical studies of isolated bacteroids (e.g. damage to membranes during isolation) were not encountered, and the physiological significance rather than just the presence of the enzymes could be assessed. The approach can now be logically extended to the isolation of mutants defective in the permease(s) for the tricarboxylic acid cycle intermediates in order that the actual energy source(s) received by the bacteroids might be identified unambiguously. This work will have to be done in conjunction with studies on isolated bacteroids in order to determine whether the same permease(s) are involved in the transport of the intermediates in free-living and bacteroid forms of rhizobia.

REFERENCES

AKKERMANS, A.D.L., ABDULKADIR, S. and TRINICK, M.J. (1978), Nitrogen-fixing root nodules in Ulmaceae, Nature, 274, 190.

ALBERSHEIM, P., AYERS, A.R., VALENT, B.S., EBEL, J., HAHN, M., WOLPERT, J. and CARLSON, R. (1977), Plants interact with microbial polysaccharides, Journal of Supramolecular Structure, 6, 599-616.

ALLISON, F.E. (1935), Carbohydrate as a primary factor in legume symbiosis, Soil Science, 39, 123-143.

ALPER, M.D. and AMES, B.N. (1978), Transport of antibiotics and metabolite analogues by systems under cyclic AMP control: positive selection of Salmonella typhimurium cya and crp mutants, Journal of Bacteriology, 133, 149-157.

AMARAL, D. and KORNBERG, H.L. (1975), Regulation of fructose uptake by glucose in Escherichia coli, Journal of General Microbiology, 90 157-168.

ANDERSON, R.L. and KAMAL, M.Y. (1966), Glucokinase. II. Aerobacter aerogenes, Methods in Enzymology, 9, 388-392.

ANDERSON, K., SHANMUGAM, K.T. and VALENTINE, R.C. (1977) Nitrogen fixation (nif) regulatory mutants of Klebsiella: determination of the energy cost of N_2 fixation in vivo, in 'Genetic engineering for nitrogen fixation', pp 95-107. Ed. A. Hollaender, New York and London, Plenum Press.

APPLEBY, C.A. (1969), Electron transport systems of Rhizobium japonicum. I. Haemoprotein P-450, other coreactive pigments, cytochromes and oxidases in bacteroids from N_2-fixing root nodules, Biochimica et Biophysica Acta, 172, 71-87.

APPLEBY, C.A., TURNER, G.L. and MacNICOL, P.K. (1975), Involvement of oxyleghaemoglobin and cytochrome P-450 in an efficient oxidative phosphorylation pathway which supports nitrogen fixation in Rhizobium, Biochimica et Biophysica Acta, 387, 461-474.

ARTHUR, L.O., BULLA, L.A., JULIAN, G.S. and NAKAMURA, L.K. (1973), Carbohydrate metabolism in Agrobacterium tumefaciens, Journal of Bacteriology, 116, 304-313.

ARTMAN, M. and WERTHAMER, S. (1974), Use of streptomycin and cyclic adenosine 3',5'-monophosphate in the isolation of mutants deficient in CAP protein, Journal of Bacteriology, 120, 542-544.

BACH, M.K., MAGEE, W.E. and BURRIS, R.H. (1958), Translocation of photosynthetic products to soybean nodules and their role in nitrogen fixation, Plant Physiology, 33, 118-124.

BAL, A.K., SHANTHARAM, S. and RATNAM, S. (1978), Ultrastructure of Rhizobium japonicum in relation to its attachment to root hairs, Journal of Bacteriology, 133, 1393-1400.

BAUER, W.D. (1977), Lectins as determinants of specificity in legume-Rhizobium symbiosis, in 'Genetic engineering for nitrogen fixation', pp 283-295. Ed. A. Hollaender, New York and London, Plenum Press.

BENDER, R.A., JANSSEN, K.A., RESNICK, A.D., BLUMENBERG, M., FOOR, F. and MAGASANIK, B. (1977), Biochemical parameters of glutamine synthetase from Klebsiella aerogenes, Journal of Bacteriology, 129, 1001-1009.

BENDER, R.A. and MAGASANIK, B. (1977), Regulatory mutations in the Klebsiella aerogenes structural gene for glutamine synthetase, Journal of Bacteriology, 132, 100-105.

BENEMANN, J.R., YOCH, D.C., VALENTINE, R.C. and ARNON, D.I. (1971), The electron transport system in nitrogen fixation by Azotobacter. III. Requirements for NADPH-supported nitrogenase activity, Biochimica et Biophysica Acta, 226, 205-212.

BENEMANN, J.R. and VALENTINE, R.C. (1972), The pathways of nitrogen fixation, Advances in Microbial Physiology, 8, 59-104.

BENNAERTS, M.J. and DELEY, J. (1963), A biochemical test for crown gall bacteria, Nature, 197, 406-407.

BERBERICH, M.A. (1972), A glutamate-dependent phenotype in E. coli K12: the result of two mutations, Biochemical and Biophysical Research Communications, 47, 1498-1503.

BERGERSEN, F.J. (1955), The cytology of bacteroids from root nodules of subterranean clover (Trifolium subterraneum L.), Journal of General Microbiology, 13, 411-419.

BERGERSEN, F.J. (1965), Ammonia - an early stable product of nitrogen fixation by soybean root nodules, Australian Journal of Biological Sciences, 18, 1-9.

BERGERSEN, F.J. (1970), The quantitative relationship between nitrogen fixation and the acetylene-reduction assay, Australian Journal of Biological Sciences, 23, 1015-1025.

BERGERSEN, F.J. (1974), Formation and function of bacteroids, in 'The biology of nitrogen fixation'. Ed. A. Quispel, pp 473-498, Amsterdam and Oxford, North-Holland.

BERGERSEN, F.J. (1977a), Nitrogenase in chemostat cultures of rhizobia, in 'Recent developments in nitrogen fixation'. Ed. W. Newton, J.R. Postgate and C. Rodriguez-Barrueco, pp. 309-320, London, Academic Press.

BERGERSEN, F.J. (1977b), Physiological chemistry of dinitrogen fixation by legumes, in 'A treatise on dinitrogen fixation, Section III: Biology'. Ed. R.W.F. Hardy and W.S. Silver, pp 519-556, New York, Wiley-Interscience.

BERGERSEN, F.J. and GOODCHILD, D.J. (1973), Cellular location and concentration of leghaemoglobin in soybean root nodules, Australian Journal of Biological Sciences, 36, 741-756.

BERGERSEN, F.J. and TURNER, G.L. (1967), Nitrogen fixation by the bacteroid fraction of breis of soybean root nodules, Biochimica et Biophysica Acta, 141, 507-515.

BERGERSEN, F.J. and TURNER, G.L. (1973), Kinetic studies of nitrogenase from soya-bean root-nodule bacteroids, Biochemical Journal, 131, 61-75.

BERGERSEN, F.J. and TURNER, G.L. (1975a), Leghaemoglobin and the supply of O_2 to nitrogen-fixing root nodule bacteroids: studies of an experimental system with no gas phase, Journal of General Microbiology, 89, 31-47.

BERGERSEN, F.J. and TURNER, G.L. (1975b), Leghaemoglobin and the supply of oxygen to nitrogen-fixing root nodule bacteroids: presence of two oxidase systems and ATP production at low free O_2 concentration, Journal of General Microbiology, 91, 345-354.

BERGERSEN, F.J. and TURNER, G.L. (1976), The role of O_2-limitation in control of nitrogenase in continuous cultures of Rhizobium sp., Biochemical and Biophysical Research Communications, 73, 524-531.

BERGERSEN, F.J., TURNER, G.L., GIBSON, A.H. and DUDMAN, W.F. (1976), Nitrogenase activity and respiration of cultures of Rhizobium spp. with special reference to concentration of dissolved oxygen, Biochimica et Biophysica Acta, 444, 164-174.

BERINGER, J.E. (1973), Genetic studies with Rhizobium leguminosarum, Ph.D. Thesis, University of Edinburgh.

BERINGER, J.E. (1974), R factor transfer in Rhizobium leguminosarum, Journal of General Microbiology, 84, 188-198.

BERINGER, J.E. and HOPWOOD, D.A. (1976), Chromosomal recombination and mapping in Rhizobium leguminosarum, Nature, 264, 291-293.

BERINGER, J.E., JOHNSTON, A.W.B. and WELLS, B. (1977), The isolation of conditional ineffective mutants of Rhizobium leguminosarum, Journal of General Microbiology, 98, 339-343.

BERINGER, J.E., HOGGAN, S.A. and JOHNSTON, A.W.B. (1978), Linkage mapping in Rhizobium leguminosarum by means of R plasmid-mediated recombination, Journal of General Microbiology, 104, 201-207.

BETHLENFALVAY, G.J. and PHILLIPS, D.A. (1977), Effect of light intensity on efficiency of carbon dioxide and nitrogen reduction in Pisum sativum L., Plant Physiology, 60, 868-871.

BHUVANESWARI, T.V., PUEPPKE, S.G. and BAUER, W.D. (1977), Role of lectins in plant-microorganism interactions. I. Binding of soybean lectin to rhizobia, Plant Physiology, 60, 486-491.

BHUVANESWARI, T.V. and BAUER, W.D. (1978), Role of lectins in plant-microorganism interactions. III. Influence of rhizosphere/rhizoplane culture conditions on the soybean lectin-binding properties of rhizobia, Plant Physiology, 62, 71-74.

BISHOP, P.E., GUEVARA, J.G., ENGELKE, J.A. and EVANS, H.J. (1976), Relation between glutamine synthetase and nitrogenase activities in the symbiotic association between Rhizobium japonicum and Glycine max, Plant Physiology, 57, 542-546.

BISSELING, T., van den BOS, R.C., van KAMMEN, A., van der PLOEG, M, van DUIJN, P. and HOUWERS, A. (1977), Cytofluorometrical determination of the DNA contents of bacteroids and corresponding broth-cultured rhizobium bacteria, Journal of General Microbiology, 101, 79-84.

BLEVINS, W.T., FEARY, T.W. and PHIBBS, P.V. (1975), 6-Phosphogluconate dehydratase deficiency in pleiotropic carbohydrate-negative mutant strains of Pseudomonas aeruginosa. Journal of Bacteriology, 121, 942-949.

BOHLOOL, B.B.and SCHMIDT, E.L. (1974), Lectins: a possible basis for specificity in the Rhizobium-legume root nodule symbiosis, Science, 185, 269-271.

BOND, G. (1941), Symbiosis of leguminous plants and nodule bacteria. I. Observations on respiration and on the extent of utilization of host carbohydrates by the nodule bacteria, Annals of Botany, 5, 313-337.

BRENCHLEY, J.E. and MAGASANIK, B. (1974), Mutants of Klebsiella aerogenes lacking glutamate dehydrogenase, Journal of Bacteriology, 117, 544-550.

BRENCHLEY, J.E., PRIVAL, M.J. and MAGASANIK, B. (1973), Regulation of the synthesis of enzymes responsible for glutamate formation in Klebsiella aerogenes, Journal of Biological Chemistry, 248, 6122-6128.

BROWN, C.M. and DILWORTH, M.J. (1975), Ammonia assimilation by Rhizobium cultures and bacteroids, Journal of General Microbiology, 86, 39-48.

BRUSSEL, A.A.N. van, PLANQUÉ, K and QUISPEL, A. (1977), The wall of Rhizobium leguminosarum in bacteroid and free-living forms, Journal of General Microbiology, 101, 51-56.

BURNS, R.C. and HARDY, R.W.F. (1975), Nitrogen fixation in bacteria and higher plants, New York, Springer-Verlag.

BURRIS, R.H. (1977), A synthesis paper on nonleguminous N_2-fixing systems, in 'Recent developments in nitrogen fixation'. Ed. W. Newton, J.R. Postgate and C. Rodriguez-Barrueco, pp 487-511, London, Academic Press.

BUTLER, G.W., GREENWOOD, R.M. and SOPER, K. (1959), Effects of shading and defoliation on the turnover of root and nodule tissue of plants of Trifolium repens, Trifolium pratense and Lotus uliginosus, New Zealand Journal of Agricultural Research, 2, 415-426.

CALDWELL, B.E. and VEST, H.G. (1977), Genetic aspects of nodulation and dinitrogen fixation by legumes: the macrosymbiant, in 'A treatise on dinitrogen fixation. Section III: Biology', Ed. R.W.F. Hardy and W.S. Silver, pp 557-576, New York and London, Wiley-Interscience.

CÁNOVAS, J.L. and KORNBERG, H.L. (1969), Phosphenolpyruvate carboxylase from Escherichia coli, Methods in Enzymology, 13, 288-292.

CARTER, K.R., JENNINGS, N.T., HANUS, J. and EVANS, H.J. (1978), Hydrogen evolution and uptake by nodules of soybeans inoculated with different strains of Rhizobium japonicum, Canadian Journal of Microbiology, 24, 307-311.

CASTRO, L., FEUCHT, B.U., MORSE, M.L. and SAIER, M.H. (1976), Regulation of carbohydrate permeases and adenylate cyclase in Escherichia coli. Studies with mutant strains in which enzyme I of the phosphoenolpyruvate:sugar phosphotransferase system is thermolabile, Journal of Biological Chemistry, 251, 5522-5527.

CHATEL, D.L., GREENWOOD, R.M. and PARKER, C.A. (1968), Saprophytic competence as an important character in the selection of Rhizobium for inoculation, Transactions of the Ninth International Congress of Soil Science, Vol. 2, pp 65-73.

CHEN, J-S. and BLANCHARD, D.K. (1978), Isolation and properties of a unidirectional H_2-oxidising hydrogenase from the strictly anaerobic N_2-fixing bacterium Clostridium pasteurianum W5, Biochemical and Biophysical Research Communications, 84, 1144-1150.

CHERN, C., KUSAKA, I. and FUKUI, S. (1976), Significance of pyruvate carboxylase in sugar metabolism of Agrobacterium tumefaciens, Agricultural and Biological Chemistry, 40, 771-778.

CHILD, J.J. (1975), Nitrogen fixation by a Rhizobium sp. in association with non-leguminous plant cell cultures, Nature, 253, 350-351.

CHILD, J.J. and LA RUE, T.A. (1974), A simple technique for the establishment of nitrogenase in soybean callus culture, Plant Physiology, 53, 88-90.

CHRISTELLER, J.T., LAING, W.T. and SUTTON, W.D. (1977), Carbon dioxide fixation by legume root nodules. I. Characterization, association with phosphoenolpyruvate carboxylase, and correlation with nitrogen fixation during nodule development, Plant Physiology, 60, 47-50.

CHUNG, A.E. and FRANZEN, J.S. (1969), Oxidised triphosphopyridine nucleotide specific isocitrate dehydrogenase from Azotobacter vinelandii, Biochemistry, 8, 3175-3184.

CÁNOVAS, J.L. and KORNBERG, H.L. (1969), Phosphenolpyruvate carboxylase from Escherichia coli. Methods in Enzymology, 13, 288-292.

CARTER, K.R., JENNINGS, N.T., HANUS, J. and EVANS, H.J. (1978), Hydrogen evolution and uptake by nodules of soybeans inoculated with different strains of Rhizobium japonicum. Canadian Journal of Microbiology, 24, 307-311.

CASTRO, L., FEUCHT, B.U., MORSE, M.L. and SAIER, M.H. (1976), Regulation of carbohydrate permeases and adenylate cyclase in Escherichia coli. Studies with mutant strains in which enzyme I of the phosphoenolpyruvate:sugar phosphotransferase system is thermolabile, Journal of Biological Chemistry, 251, 5522-5527.

CHATEL, D.L., GREENWOOD, R.M. and PARKER, C.A. (1968), Saprophytic competence as an important character in the selection of Rhizobium for inoculation, Transactions of the Ninth International Congress of Soil Science, Vol. 2, pp 65-73.

CHEN, J-S. and BLANCHARD, D.K. (1978), Isolation and properties of a unidirectional H_2-oxidising hydrogenase from the strictly anaerobic N_2-fixing bacterium Clostridium pasteurianum W5, Biochemical and Biophysical Research Communications, 84, 1144-1150.

CHERN, C., KUSAKA, I. and FUKUI, S. (1976), Significance of pyruvate carboxylase in sugar metabolism of Agrobacterium tumefaciens, Agricultural and Biological Chemistry, 40, 771-778.

CHILD, J.J. (1975), Nitrogen fixation by a Rhizobium sp. in association with non-leguminous plant cell cultures, Nature, 253, 350-351.

CHILD, J.J. and LA RUE, T.A. (1974), A simple technique for the establishment of nitrogenase in soybean callus culture, Plant Physiology, 53, 88-90.

CHRISTELLER, J.T., LAING, W.T. and SUTTON, W.D. (1977), Carbon dioxide fixation by legume root nodules. I. Characterization, association with phosphoenolpyruvate carboxylase, and correlation with nitrogen fixation during nodule development, Plant Physiology, 60, 47-50.

CHUNG, A.E. and FRANZEN, J.S. (1969), Oxidised triphosphopyridine nucleotide specific isocitrate dehydrogenase from Azotobacter vinelandii, Biochemistry, 8, 3175-3184.

CLARKE, P.H. and ORNSTON, L.N. (1975), Metabolic pathways and regulation, I. In 'Genetics and Biochemistry of Pseudomonas', Ed. P.H. Clarke and M.H. Richmond, pp 191-261, London, Wiley-Interscience.

COLE, J.A., COLEMAN, K.J., COMPTON, B.E., KAVANAGH, B.M. and KEEVIL, C.W. (1974), Nitrate and ammonia assimilation by anaerobic continuous cultures of Escherichia coli, Journal of General Microbiology, 85, 11-22.

COOPER, R.A. (1978), The utilisation of D-galactonate and D-2-oxo-3-deoxygalactonate by Escherichia coli K12, Biochemical and genetical studies, Archives for Microbiology, 118, 199-206.

COVENTRY, D.R., TRINICK, M.J. and APPLEBY, C.A. (1976), A search for a leghaemoglobin-like compound in root nodules of Trema cannabina Lour, Biochimica et Biophysica Acta, 420, 105-111.

CUTTING, J.A. and SCHULMAN, H.M. (1969), The site of heme synthesis in soybean nodules, Biochimica et Biophysica Acta, 192, 486-493.

DAHMS, R.S. and ANDERSON, R.L. (1969), 2-Keto-3-deoxy-L-arabonate aldolase and its role in a new pathway of L-arabinose degradation, Biochemical and Biophysical Research Communications, 36, 809-814.

DALTON, H. (1974), Fixation of dinitrogen by free-living microorganisms, Critical Reviews in Microbiology, 3, 183-220.

DALTON, H. and POSTGATE, J.R. (1969), Growth and physiology of Azotobacter chroococcum in continuous culture, Journal of General Microbiology, 56, 307-319.

DARROW, R.A. and KNOTTS, R.R. (1977), Two forms of glutamine synthetase in free-living root-nodule bacteria, Biochemical and Biophysical Research Communications, 78, 554-559.

DART, P. (1977), Infection and development of leguminous nodules, in 'A treatise on dinitrogen fixation, Section III: Biology', Ed. R.W.F. Hardy and W.S. Silver, pp 367-472, New York, Wiley-Interscience.

DAVIS, L.D. and ORME-JOHNSON, W.H. (1976), Nitrogenase IX. Effect of the Mg.ATP generator on the catalytic and EPR properties of the enzyme in vitro, Biochimica et Biophysica Acta, 452, 42-58.

DAWES, E.A. and SENIOR, P.J. (1973), The role and regulation of energy reserve polymers in microorganisms, Advances in Microbial Physiology, 10, 135-266.

DAZZO, F.B. and BRILL, W.J. (1977), Receptor site on clover and alfalfa roots for Rhizobium, Applied and Environmental Microbiology, 33, 132-136.

DAZZO, F.B. and BRILL, W.J. (1978), Regulation by fixed nitrogen of host-symbiont recognition in the Rhizobium-clover symbiosis, Plant Physiology, 62, 18-21.

DAZZO, F.B. and HUBBELL, D.H. (1975), Cross-reactive antigens and lectin as determinants of symbiotic specificity in the Rhizobium-clover association, Applied Microbiology, 30, 1017-10[illegible]

DAZZO, F.B., NAPOLI, C.A. and HUBBELL, D.H. (1976) Adsorption of bacteria to roots as related to host specificity in the Rhizobium-clover symbiosis, Applied and Environmental Microbiology, 32, 166-171.

DAZZO, F.B., YANKE, W.E. and BRILL, W.J. (1978), Trifoliin: a Rhizobium recognition protein from white clover, Biochimica et Biophysica Acta, 539, 276-286.

DEACON, J. and COOPER, R.A. (1977), D-galactonate utilisation by enteric bacteria: the catabolic pathway in Escherichia coli, FEBS Letters, 77, 201-205.

DECKER, S.J. and LANG, D.R. (1977) Bacillus megaterium mutant deficient in membrane-bound adenosine triphosphatase activity, Journal of Bacteriology, 131, 98-104.

DeLEY, J. and DOUDOROFF, M. (1957), The metabolism of D-galactose in Pseudomonas saccharophila, Journal of Biological Chemistry, 227, 745-757.

DÉNARIÉ, J., TRUCHET, G. and BERGERON, B. (1976), Effects of some mutations on symbiotic properties of Rhizobium, in 'Symbiotic nitrogen fixation in plants', Ed. P.S. Nutman, pp 47-62, Cambridge, Cambridge University Press.

DILWORTH, M.J. (1969), The plant as the genetic determinant of leg-haemoglobin production in the legume root nodule, Biochimica et Biophysica Acta, 184, 432-441.

DILWORTH, M.J. and KIDBY, D.K. (1968), Localization of iron and leghaemoglobin in the legume root nodule by electron microscope autoradiography, Experimental Cell Research, 49, 148-159.

DILWORTH, M.J. and WILLIAMS, D.C. (1967), Nucleic acid changes in bacteroids of Rhizobium lupini during nodule development, Journal of General Microbiology, 48, 31-36.

DIXON, R.O.D. (1967), Hydrogen uptake and exchange by pea root nodules, Annals of Botany, 31, 179-188.

DIXON, R.O.D. (1972), Hydrogenase in legume root nodule bacteroids: occurrence and properties, Archiv für Mikrobiologie, 85, 193-201.

DOELLE, H.W. (1969), Chemosynthesis. Pathways of carbohydrate metabolism, in 'Bacterial metabolism', Ed. H.W. Doelle, pp 129-198, New York: Academic Press.

DUNICAN, L.K., O'GARA, F. and TIERNEY, A.B. (1976), Plasmid control of effectiveness in Rhizobium: transfer of nitrogen-fixing genes on a plasmid from Rhizobium trifolii to Klebsiella aerogenes, in 'Symbiotic nitrogen fixation in plants', Ed. P.S. Nutman, pp 77-90, Cambridge: Cambridge University Press.

DUNN, S.D. and KLUCAS, R.V. (1973), Studies on possible routes of ammonium assimilation in soybean root nodule bacteroids, Canadian Journal of Microbiology, 19, 1493-1499.

EADY, R.R., SMITH, B.E., COOK, K.A. and POSTGATE, J.R. (1972) Nitrogenase of Klebsiella pneumoniae. Purification and properties of the component proteins, Biochemical Journal, 128, 655-675.

EADY, R.R. and POSTGATE, J.R. (1974), Nitrogenase, Nature, 249, 805-810.

EADY, R.R. and SMITH, B.E. (1978), Physico-chemical properties of nitrogenase and its component proteins, in 'A treatise on dinitrogen fixation. Section II: Biochemistry', Ed. R.W.F. Hardy and R.C. Burns, in the press. New York: Wiley-Interscience.

EADY, R.R., ISAACK, R., KENNEDY, C., POSTGATE, J.R. and RATCLIFFE, H.D. (1978a), Nitrogenase synthesis in Klebsiella pneumoniae: comparison of ammonium and oxygen regulation, Journal of General Microbiology, 104, 277-285.

EADY, R.R., LOWE, D.J. and THORNELEY, R.N.F. (1978b), Nitrogenase of Klebsiella pneumoniae: a pre-steady state burst of ATP hydrolysis is coupled to electron transfer between the component proteins, FEBS Letters, 95, 211-213.

ELLIOT, W.H. (1955), Glutamine synthesis, Methods in Enzymology, 2, 337-342.

ENGLARD, S. and SIEGEL, L. (1969), Mitochondrial L-malate dehydrogenase of beef heart, Methods in Enzymology, 13, 99-106.

ENTIAN, K.D. (1977), Lack of carbon catabolite inactivation in a mutant of Saccharomyces cerevisiae with reduced hexokinase activity, Molecular and General Genetics, 158, 201-210.

EVANS, H.J. and PHILLIPS, D.A. (1975), Reductants for nitrogenase and relationships to cellular electron transport, in 'Nitrogen fixation by free-living microorganisms', Ed. W.D.P. Stewart, pp 389-420, Cambridge: Cambridge University Press.

FLAKS, J.G. and LUKENS, L.N. (1963), The enzymes of purine nucleotide synthesis de novo, Methods in Enzymology, 6, 52-95.

FOOR, F., JANSSEN, K.A. and MAGASANIK, B. (1975), Regulation of synthesis of glutamine synthetase by adenylylated glutamine synthetase, Proceedings of the National Academy of the United States of America, 72, 4844-4848.

FRADKIN, J.E. and FRAENKEL, D.G. (1971), 2-Keto-3-deoxygluconate 6-phosphate aldolase mutants of Escherichia coli, Journal of Bacteriology, 108, 1277-1283.

FRAENKEL, D.G. and VINOPAL, R.T. (1973), Carbohydrate metabolism in bacteria, Annual Review of Microbiology, 27, 69-100.

FRED, E.B., BALDWIN, I.L. and McCOY, E. (1932), Root nodule bacteria and leguminous plants, University of Wisconsin's Studies in Science, No. 5, Madison.

FUNANAGE, V.L., AYLING, P.D., DENDINGER, S.M. and BRENCHLEY, J.E. (1978), Salmonella typhimurium LT-2 mutants with altered glutamine synthetase levels and amino acid uptake activities, Journal of Bacteriology, 136, 588-596.

GIBBINS, A.M. and GREGORY, K.F. (1972), Relatedness among Rhizobium and Agrobacterium species determined by three methods of nucleic acid hybridization, Journal of Bacteriology, 111, 129-141.

GIBSON, A.H. (1966), The carbohydrate requirements for symbiotic nitrogen fixation: a "whole-plant" growth analysis approach, Australian Journal of Biological Science, 19, 499-515.

GIBSON, A.H. (1976), Recovery and compensation by nodulated legumes to environmental stress, in 'Symbiotic nitrogen fixation in plants; International biological programme, Vol. 7. Ed. P.S. Nutman, pp 385-403, Cambridge: Cambridge University Press.

GIBSON, A.H., SCOWCROFT, W.R. and PAGAN, J.D. (1977), Nitrogen fixation in plants: an expanding horizon ?, in 'Recent developments in nitrogen fixation'. Ed. W. Newton, J.R. Postgate and C. Rodriguez-Barrueco, pp 387-418, London: Academic Press.

GINSBURG, A. and STADTMAN, E.R. (1973), Regulation of glutamine synthetase in Escherichia coli, in 'The enzymes of glutamine metabolism'. Ed. S. Prusiner and E.R. Stadtman, pp 9-43, New York: Academic Press.

GODFREY, C.A., COVENTRY, D.R. and DILWORTH, M.J. (1975), Some aspects of leghaemoglobin biosynthesis, in 'Nitrogen fixation by free-living microorganisms'. Ed. W.D.P. Stewart, pp 311-332, Cambridge: Cambridge University Press.

GONZALEZ, J.E. and PETERKOFSKY, A. (1977), The mechanism of sugar-dependent repression of synthesis of catabolic enzymes in Escherichia coli, Journal of Supramolecular Structure, 6, 495-502.

GOURRET, J-P. and FERNANDEZ-ARIAS, H. (1974), Étude ultra-structurale et cytochimique de la différenciation des bactéroïdes de Rhizobium trifolii Dangeard dans les nodules de Trifolium repens L, Canadian Journal of Microbiology, 20, 1169-1181.

GRAHAM, P.H. (1964), Studies on the utilisation of carbohydrates and Kreb's cycle intermediates by rhizobia, using an agar plate method, Antonie van Leeuwenhoek, Journal of Microbiology and Serology, 30, 68-72.

GRAHAM, P.H.(1976), Identification and classification of root nodule bacteria, in 'Symbiotic nitrogen fixation in plants'. Ed. P.S. Nutman, pp 99-112, Cambridge: Cambridge University Press.

GRESSHOFF, P.M., SKOTNICKI, M.L., EADIE, J.F. and ROLFE, B.G.(1977), Viability of Rhizobium trifolii bacteroids from clover root nodules, Plant Science Letters, 10, 299-304.

GRESSHOFF, P.M. and ROLFE, B.G. (1978), Viability of Rhizobium bacteroids isolated from soybean nodule protoplasts, Planta, 142, 329-333.

HAAKER, H., DEKOK, A. and VEEGER, C. (1974), Regulation of dinitrogen fixation in intact Azotobacter vinelandii, Biochimica et Biophysica Acta, 357, 344-357.

HAAS, D. and HOLLOWAY, B.W. (1976), R factor variants with enhanced sex factor activity in Pseudomonas aeruginosa, Molecular and General Genetics, 144, 243-251.

HAM, G.E., LAWN, R.J. and BRUN, W.A. (1976), Influence of inoculation, nitrogen fertilizers and photosynthetic source-sink manipulations on field-grown soybeans, in 'Symbiotic nitrogen fixation in plants: International biological programme, Vol. 7.' Ed. P.S. Nutman, pp 239-253, Cambridge: Cambridge University Press.

HARDY, R.W.F. and GIBSON, A.H. (eds.) (1977), 'A Treatise on Dinitrogen Fixation, Section IV: Agronomy and Ecology.' New York, Wiley-Interscience.

HARDY, R.W.F. and HAVELKA, U.D. (1976), Photosynthate as a major factor limiting nitrogen fixation by field-grown legumes, with emphasis on soybeans, in 'Symbiotic nitrogen fixation in plants: International biological programme, Vol. 7.' Ed. P.S. Nutman, pp 421-439, Cambridge: Cambridge University Press.

HARDY, R.W.F. and SILVER, W.D. (eds.) (1977). 'A Treatise on Dinitrogen Fixation, Section III; Biology'. New York, Wiley-Interscience.

HAUGE, J.G. (1966), Glucose dehydrogenases-soluble II. Bacterium anitratum, Methods in Enzymology, 9, 107-112.

HERBERT, D., PHIPPS, D.J., and STRANGE, R.E. (1971), Chemical analysis of microbial cells, Methods in Microbiology, 5B, 209-344.

HERRIDGE, D.F. and PATE, J.S. (1977), Utilisation of net photosynthate for nitrogen fixation and protein production in an annual legume, Plant Physiology, 60, 759-764.

HIGASHI, S. (1967), Transfer of clover infectivity of Rhizobium trifolii to Rhizobium phaseoli as mediated by an episomic factor, Journal of General and Applied Microbiology, 13, 391-403.

HILL, S. (1976), The apparent ATP requirement for nitrogen fixation in growing Klebsiella pneumoniae, Journal of General Microbiology, 95, 297-312.

HOLLAENDER, A. (ed.) (1977). 'Genetic Engineering for Nitrogen Fixation'. New York and London: Plenum Press.

HOLLIDAY, R. (1956), A new method for the identification of biochemical mutants of microorganisms, Nature, 178, 987.

HOOYKAAS, P.J.J., KLAPWIJK, P.M., NUTI, M.P., SCHILPEROORT, R.A. and RORSCH, A. (1977), Transfer of the Agrobacterium tumefaciens TI plasmid to avirulent Agrobacteria and to Rhizobium ex planta, Journal of General Microbiology, 98, 477-484.

HSU, R.Y. and LARDY, H.A. (1969), Malic enzyme, Methods in Enzymology, 13, 230-235.

HYNDMAN, L.A., BURRIS, R.H. and WILSON, P.W. (1953), Properties of hydrogenase from Azotobacter vinelandii, Journal of Bacteriology, 65, 522-531.

ISRAEL, D.W., HOWARD, R.L., EVANS, H.J. and RUSSEL, S.A. (1974), Purification and characterisation of the molybdenum-iron protein component of nitrogenase from soybean nodule bacteroids, Journal of Biological Chemistry, 249, 500-508.

JACOB, A.E., CRESSWELL, J.M., HEDGES, R.W., COETZEE, J.N. and BERINGER, J.E. (1976), Properties of plasmids constructed by the in vitro insertion of DNA from Rhizobium leguminosarum or Proteus mirabilis into RP4, Molecular and General Genetics, 147, 315-323.

JACOB, A.E., CRESSWELL, J.M. and HEDGES, R.W. (1977) Molecular characterisation of the F group plasmid R68 and variants with enhanced chromosome mobilizing ability, FEMS Microbiology Letters, 1, 71-74.

JOHNSON, G.V., EVANS, H.J. and CHING, T. (1966), Enzymes of the glyoxalate cycle in rhizobia and nodules of legumes, Plant Physiology, 41, 1330-1336.

JOHNSTON, A.W.B. and BERINGER, J.E. (1977), Chromosomal recombination between Rhizobium species, Nature, 267, 611-613.

JOHNSTON, A.W.B., BEYNON, J.L., BUCHANAN-WOLLASTON, A.V., SETCHELL, S.M., HIRSCH, P.R. and BERINGER, J.E. (1978a), High frequency transfer of nodulating ability between strains and species of Rhizobium. Nature, 276, 634-636.

JOHNSTON, A.W.B., SETCHELL, S.M. and BERINGER, J.E. (1978b) Interspecific crosses between Rhizobium leguminosarum and R. meliloti: formation of haploid recombinants and of R. primes, Journal of General Microbiology, 104, 209-218.

JOHNSTON, A.W.B., BIBB, M.J. and BERINGER, J.E. (1978c), Tryptophan genes in Rhizobium - their organization and their transfer to other bacterial genera. Molecular and General Genetics, 165, 323-330.

JORDAN, D.C. (1962), The bacteroids of the genus Rhizobium, Bacteriological Reviews, 26, 119-141.

KATZNELSON, H. and ZAGALLO, A.C. (1957), Metabolism of rhizobia in relation to effectiveness, Canadian Journal of Microbiology, 3, 879-884.

KEELE, B.B., HAMILTON, P.B. and ELKAN, G.H. (1969), Glucose catabolism in Rhizobium japonicum, Journal of Bacteriology, 97, 1184-1191.

KEELE, B.B., HAMILTON, P.B. and ELKAN, G.H. (1970), Gluconate catabolism in Rhizobium japonicum, Journal of Bacteriology, 101, 698-704.

KEISTER, D.L. (1975), Acetylene reduction by pure cultures of rhizobia, Journal of Bacteriology, 123, 1265-1268.

KENNEDY, C., EADY, R.R., KONDOROSI, E. and KLAVINS-REKOSH, D. (1976), The molybdenum-iron protein of Klebsiella pneumoniae: evidence for non-identical subunits from peptide mapping, Biochemical Journal, 155, 383-389.

KIDBY, D.K. (1966), Activation of a plant invertase by inorganic phosphate, Plant Physiology, 41, 1139-1144.

KIDBY, D.K. (1967), Carbon metabolism in legume root nodules, Ph.D. Thesis, University of Western Australia.

KLEIN, G.E., JEMISON, P. HAAK, R.A. and MATHYSSE, A. (1975), Physical evidence of a plasmid in Rhizobium japonicum, Experientia, 31, 532-533.

KONDOROSI, Á., BARABÁS, I., SVÁB, Z., OROSZ, L., SIK, T. and HOTCHKISS, R.D. (1973), Evidence for common genetic determinants of nitrogenase and nitrate reductase in Rhizobium meliloti, Nature New Biology, 246, 153-154.

KONDOROSI, Á., SVÁB, Z., KISS, G.B. and DIXON, R.A. (1977a), Ammonia assimilation and nitrogen fixation in Rhizobium meliloti, Molecular and General Genetics, 151, 221-226.

KONDOROSI, Á, KISS, G.B., FORRAI, T., VINCZE, E. and BANFALVI, Z. (1977b), Circular linkage map of Rhizobium meliloti chromosome, Nature, 268, 525-527.

KORNBERG, H.L. (1966), Anaplerotic sequences and their role in metabolism, in 'Essays in biochemistry', Vol. 2. Ed. P.N. Campbell and G.D. Greville, pp 1-31, London and New York: Academic Press.

KORNBERG, H.L. and JONES-MORTIMER, M.C. (1977), The phosphotransferase system as a site of cellular control, Symposia of the Society for General Microbiology, 27, 217-240.

KORNBERG, H.L. and RIORDAN, C. (1976), Uptake of galactose into Escherichia coli by facilitated diffusion, Journal of General Microbiology, 94, 75-89.

KOWALSKI, M. and DÉNARIÉ, J. (1972), Transduction d'un gène contrôlant l'expression de la fixation de l'azote chez Rhizobium meliloti, Comptes rendus de l'Academie des Sciences, Paris, Ser. D., 275, 141-144.

KRETOVICH, W.L., ROMANOV, V.I., YUSHKOVA, L.A., SHRAMKO, V.I. and FEDULOVA N.G. (1977), Nitrogen fixation and poly-β-hydroxybutyric acid content in bacteroids of Rhizobium lupini and Rhizobium leguminosarum, Plant and Soil, 48, 291-302.

KUBY, S.A. and NOLTMAN, E.A. (1966), Glucose 6-phosphate dehydrogenase (crystalline) from Brewer's yeast, Methods in Enzymology, 9, 116-125.

KUMAR, S. (1976), Properties of adenyl cyclase and cyclic adenosine 3',5'-monophosphate receptor protein-deficient mutants of Escherichia coli. Journal of Bacteriology, 125, 545-555.

KURZ, W.G.W. and LA RUE, T.A. (1975), Nitrogenase activity in rhizobia in the absence of plant host, Nature, 256, 407-409.

KURZ, W.G.W. and LA RUE, T.A. (1977), Citric acid cycle enzymes and nitrogenase in nodules of Pisum sativum. Canadian Journal of Microbiology, 23, 1197-1200.

KURZ, W.G.W., ROKOSH, D.A. and LA RUE T.A. (1975), Enzymes of ammonia assimilation in Rhizobium leguminosarum bacteroids, Canadian Journal of Microbiology, 21, 1009-1012.

KUYKENDALL, L.D. and ELKAN, G.H. (1977), Some features of mannitol metabolism in Rhizobium japonicum. Journal of General Microbiology, 98, 291-295.

LAANE, C., HAAKER, H. and VEEGER, C. (1978), Involvement of the cytoplasmic membrane in nitrogen fixation by Rhizobium leguminosarum bacteroids. European Journal of Biochemistry, 87, 147-154.

LABANDERA, C.A. and VINCENT, J.M. (1975), Loss of symbiotic capacity in commercially useful strains of Rhizobium trifolii. Journal of Applied Bacteriology, 39, 209-211.

LARABEKE, N. van, ENGLER, G., HOLSTERS, M., van den ELSACKER, S., ZAENEN, I., SCHILPEROORT, R.A. and SCHELL, J. (1974), Large plasmid in Agrobacterium tumefaciens essential for crown gall inducing ability, Nature, 252, 169-170.

LARABEKE, N. van, GENETELLO, C., SCHELL, J., SCHILPEROORT R.A., HERMANS, A.K., HERNALSTEENS, J.P. and van MONTAGU, M. (1975), Acquisition of tumour inducing ability by nononcogenic Agrobacteria as a result of plasmid transfer, Nature, 255, 742-743.

LAWN, R.J. and BRUN, W.A. (1974), Symbiotic nitrogen fixation in soybeans. I. Effect of photosynthetic source-sink manipulations, Crop Science, 14, 11-16.

LAWRIE, A.C. and WHEELER, C.T. (1975), Nitrogen fixation in the root nodules of Vicia faba L. in relation to the assimilation of carbon. II. The dark fixation of carbon dioxide, New Phytologist, 74, 437-445.

LESSIE, T.G. and NEIDHARDT, F.C. (1967), Formation and operation of the histidine-degrading pathway in Pseudomonas aeruginosa, Journal of Bacteriology, 93, 1800-1810.

LEVIN, R.A. and MONTGOMERY, M.P. (1974), Symbiotic effectiveness of antibiotic-resistant mutants of Rhizobium japonicum, Plant and Soil, 41, 669-676.

LIBBENGA, K.R. and BOGERS, R.J. (1974), Root-nodule morphogenesis, in 'The biology of nitrogen fixation'. Ed. A. Quispel, pp 430-472, Amsterdam and Oxford: North-Holland.

LILLICH, T.T. and ELKAN, G.H. (1971), The biosynthesis of aspartic acid, glutamic acid and alanine in Rhizobium japonicum, Canadian Journal of Microbiology, 17, 683-688.

LING, K.H., PAETKAU, V., MARCUS, F. and LARDY, H.A. (1966), Phosphofructokinase, I. Skeletal muscle, Methods in Enzymology, 9, 425-429.

LOBO, Z., and MAITRA, P.K. (1977), Resistance to 2-deoxyglucose in yeast: a direct selection of mutants lacking glucose phosphorylating enzymes, Molecular and General Genetics, 157, 297-300.

LOPEZ, J.M. and THOMS, B. (1977), Role of sugar uptake and metabolic intermediates on catabolite repression in Bacillus subtilis, Journal of Bacteriology, 129, 217-224.

LORKIEWICZ, Z., ZURKOWSKI, W., KOWALCZUK, E. and GORSKA-MELKE, A. (1971), Mutagenesis and conjugation in Rhizobium trifolii, Acta Microbiologica Polonica, Ser. A., 3, 101-107.

LUDWIG, R.A. (1978), Control of ammonium assimilation in Rhizobium 32HI, Journal of Bacteriology, 135, 114-123.

LUDWIG, R.A. and SIGNER, E.R. (1977), Glutamine synthetase and control of nitrogen fixation in Rhizobium, Nature, 267, 245-248.

McCOMB, J.A., ELLIOTT, J. and DILWORTH, M.J. (1975), Acetylene reduction by Rhizobium in pure culture, Nature, 256, 409-410.

McGINNIS, J.F. and PAIGEN, K. (1969), Catabolite inhibition: a general phenomenon in the control of carbohydrate utilization, Journal of Bacteriology, 100, 902-913.

McGINNIS, J.F. and PAIGEN, K. (1973), Site of catabolite inhibition of carbohydrate metabolism, Journal of Bacteriology, 114, 885-887.

MACKENZIE, C.R. and JORDAN, D.C. (1972), Cell wall composition and viomycin resistance in Rhizobium meliloti, Canadian Journal of Microbiology, 18, 1168-1170.

MACKENZIE, C.R., VAIL, W.J. and JORDAN, D.C. (1973), Ultra-structure of free-living and nitrogen-fixing forms of Rhizobium meliloti as revealed by freeze-etching, Journal of Bacteriology, 113, 387-393.

MAGASANIK, B. (1970), Glucose effects: inducer exclusion and repression, in ' The lactose operon'. Ed. J.R. Beckwith and D. Zipser, pp 189-219, Cold Spring Harbor: Cold Spring Harbor Laboratory.

MAGASANIK, B., LUND, P., NEIDHARDT, F.C. and SCHWARTZ, D.T. (1965), Induction and repression of the histidine-degrading enzymes in Aerobacter aerogenes, Journal of Biological Chemistry, 240, 4320-4324.

MAGASANIK, B., KAMINSKAS, E. and KIMHI, Y. (1971), Histidase (histidine ammonia-lyase) (Bacillus subtilis), Methods in Enzymology, 17B, 47-50.

MAGASANIK, B., PRIVAL, M.J., BRENCHLEY, J.E., TYLER, B.M., DELEO, A.B., STREICHER, S.L., BENDER, R.A. and PARIS, C.G. (1974), Glutamine synthetase as a regulator of enzyme synthesis, Current Topics in Cellular Regulation, 8, 119-138.

MAHON, J.D. (1977a), Root and nodule respiration in relation to acetylene reduction in intact nodulated peas, Plant Physiology, 60, 812-816.

MAHON, J.D. (1977b), Respiration and the energy requirement for nitrogen fixation in nodulated pea roots, Plant Physiology, 60, 817-821.

MAIER, R.J. and BRILL, W.J. (1976), Ineffective and non-nodulating mutant strains of Rhizobium japonicum. Journal of Bacteriology, 127, 763-769.

MAIER, R.J. and BRILL, W.J. (1978), Involvement of Rhizobium japonicum O antigen in soybean nodulation, Journal of Bacteriology, 133, 1295-1299.

MAIER, R.J., CAMPBELL, N.E.R., HANUS, F.J., SIMPSON, F.B., RUSSELL, S.A. and EVANS, H.J. (1978a), Expression of hydrogenase activity in free-living Rhizobium japonicum. Proceedings of the National Academy of Sciences of the United States of America, 75, 3258-3262.

MAIER, R.J., POSTGATE, J.R. and EVANS, H.J. (1978b), Rhizobium japonicum mutants unable to use hydrogen, Nature, 276, 494-495.

MARTINEZ-de DRETS, G. and ARIAS, A. (1970), Metabolism of some polyols by Rhizobium meliloti, Journal of Bacteriology, 103, 97-103.

MARTINEZ-de DRETS, G. and ARIAS, A. (1972), Enzymatic basis for differentiation of Rhizobium into fast- and slow-growing groups, Journal of Bacteriology, 109, 467-470.

MARTINEZ-de DRETS, G., ARIAS, A. and ROVIRA de CUTINELLA, M. (1974), Fast- and slow-growing rhizobia. Differences in sucrose utilization and invertase activity, Canadian Journal of Microbiology, 20, 605-609.

MEADE, H.M. and SIGNER, E.R. (1977), Genetic mapping of Rhizobium meliloti, Proceedings of the National Academy of Sciences of the United States of America, 74, 2076-2078.

MIDGLEY, M. and DAWES, E.A. (1973), The regulation of transport of glucose and methyl α-glucoside in Pseudomonas aeruginosa, Biochemical Journal, 132, 141-154.

MIFLIN, B.J. and LEA, P.J. (1976), The pathway of nitrogen assimilation in plants, Phytochemistry, 15, 873-885.

MINCHIN, F.R. and PATE, J.S. (1973), The carbon balance of a legume and the functional economy of its root nodules, Journal of Experimental Botany, 25, 295-308.

MINCHIN, F.R. and PATE, J.S. (1974), Diurnal functioning of the legume root nodule, Journal of Experimental Botany, 25, 295-308.

MOHAPATRA, N. and KLOOS, W.E. (1975), Biochemical characterization and genetic mapping of purine genes in Micrococcus luteus, Genetical Research, Cambridge, 26, 163-171.

MUKKADA, A.J., LONG, G.L. and ROMANO, A.H. (1973), The uptake of 2-deoxy-D-glucose by Pseudomonas aeruginosa and its regulation, Biochemical Journal, 132, 155-162.

MULONGOY, K. and ELKAN, G.H. (1977a), Glucose catabolism in two derivatives of a Rhizobium japonicum strain differing in nitrogen-fixing efficiency, Journal of Bacteriology, 131, 179-187.

MULONGOY, K. and ELKAN, G.H. (1977b), The role of 6-phosphogluconate dehydrogenase in Rhizobium, Canadian Journal of Microbiology, 23, 1293-1298.

MUMFORD, F.E., CARNAHAN, J.E. and CASTLE, J.E. (1959), Nitrogen fixation in a mutant of Azotobacter vinelandii, Journal of Bacteriology, 77, 86-90.

NADLER, K.D. and AVISSAR, Y.J. (1977), Heme synthesis in soybean root nodules, I. On the role of bacteroid δ-aminolevulinic acid synthase and δ-aminolevulinic acid dehydrase in the synthesis of the heme of leghaemoglobin, Plant Physiology, 60, 433-436.

NAGATANI, H.H. and BRILL, W.J. (1974), Nitrogenase V. The effect of Mo, W and V on the synthesis of nitrogenase components in Azotobacter vinelandii, Biochimica et Biophysica Acta, 362, 160-166.

NAGATANI, H.H., SHIMIZU, M. and VALENTINE, R.C. (1971), The mechanism of ammonia assimilation in nitrogen fixing bacteria, Archiv für Mikrobiologie, 79, 164-175.

NAGATANI, H.H., SHAH, V.K. and BRILL, W.J. (1974), Activation of inactive nitrogenase by acid-treated component 1, Journal of Bacteriology, 120, 697-701.

NASON, A., LEE, K-Y., PAN, S-S, KETCHUM, P.A., LAMBERTI, A. and DE VRIES, J. (1971), In vitro formation of assimilatory reduced nicotinamide adenine dinucleotide phosphate: nitrate-reductase from a Neurospora mutant and a component of molybdenum-enzymes, Proceedings of the National Academy of Sciences of the United States of America, 68, 3242-3246.

MINCHIN, F.R. and PATE, J.S. (1974), Diurnal functioning of the legume root nodule, Journal of Experimental Botany, 25, 295-308.

MOHAPATRA, N. and KLOOS, W.E. (1975), Biochemical characterization and genetic mapping of purine genes in Micrococcus luteus, Genetical Research, Cambridge, 26, 163-171.

MUKKADA, A.J., LONG, G.L. and ROMANO, A.H. (1973), The uptake of 2-deoxy-D-glucose by Pseudomonas aeruginosa and its regulation, Biochemical Journal, 132, 155-162.

MULONGOY, K. and ELKAN, G.H. (1977a), Glucose catabolism in two derivatives of a Rhizobium japonicum strain differing in nitrogen-fixing efficiency, Journal of Bacteriology, 131, 179-187.

MULONGOY, K. and ELKAN, G.H. (1977b), The role of 6-phosphogluconate dehydrogenase in Rhizobium, Canadian Journal of Microbiology, 23, 1293-1298.

MUMFORD, F.E., CARNAHAN, J.E. and CASTLE, J.E. (1959), Nitrogen fixation in a mutant of Azotobacter vinelandii, Journal of Bacteriology, 77, 86-90.

NADLER, K.D. and AVISSAR, Y.J. (1977), Heme synthesis in soybean root nodules, I. On the role of bacteroid δ-aminolevulinic acid synthase and δ-aminolevulinic acid dehydrase in the synthesis of the heme of leghaemoglobin, Plant Physiology, 60, 433-436.

NAGATANI, H.H. and BRILL, W.J. (1974), Nitrogenase V. The effect of Mo, W and V on the synthesis of nitrogenase components in Azotobacter vinelandii, Biochimica et Biophysica Acta, 362, 160-166.

NAGATANI, H.H., SHIMIZU, M. and VALENTINE, R.C. (1971), The mechanism of ammonia assimilation in nitrogen fixing bacteria, Archiv für Mikrobiologie, 79, 164-175.

NAGATANI, H.H., SHAH, V.K. and BRILL, W.J. (1974), Activation of inactive nitrogenase by acid-treated component 1, Journal of Bacteriology, 120, 697-701.

NASON, A., LEE, K-Y., PAN, S-S, KETCHUM, P.A., LAMBERTI, A. and DE VRIES, J. (1971), In vitro formation of assimilatory reduced nicotinamide adenine dinucleotide phosphate: nitrate-reductase from a Neurospora mutant and a component of molybdenum-enzymes, Proceedings of the National Academy of Sciences of the United States of America, 68, 3242-3246.

NEIDHARDT, F.C. and MAGASANIK, B. (1957), Reversal of the glucose inhibition of histidase biosynthesis in Aerobacter aerogenes. Journal of Bacteriology. 73. 253-259.

NEWTON, W.E. and NYMAN, C.E. (eds.)(1976). 'Proceedings of the First International Symposium on Nitrogen Fixation' Pullman: Washington State Press.

NEWTON, W.E., POSTGATE, J.R. and RODRIGUEZ-BARRUECO, C. (eds.)(1977). 'Recent Developments in Nitrogen Fixation'. London: Academic Press.

NG, F.M.W. and DAWES, E.A. (1973), Chemostat studies on the regulation of glucose metabolism in Pseudomonas aeruginosa by citrate, Biochemical Journal. 132. 129-140.

NIEL, C., GUILLAUME, J.B. and BECHET, M. (1977), Mise en évidence de deux enzymes présentant une activité β-galactosidasique chez Rhizobium meliloti. Canadian journal of Microbiology. 23. 1178-1181.

NOLTMAN, E.A. (1966), Phosphoglucose isomerase I. Rabbit muscle (crystalline), Methods in Enzymology. 9. 557-568.

NUTI, M.P., LEDEBOER, A.M., LEPIDI, A.A. and SCHILPEROORT, R.A. (1977), Large plasmids in different Rhizobium species, Journal of General Microbiooogy. 100. 241-248.

NUTMAN, P.S. (ed.)(1976). 'Symbiotic Nitrogen Fixation in Plants'. Cambridge: Cambridge University Press.

O'GARA, F. and SHANMUGAM, K.T. (1976), Regulation of nitrogen fixation by rhizobia. Export of fixed N_2 as NH_4^+, Biochimica et Biophysica Acta. 437. 313-321.

O'GARA, F. and SHANMUGAM, K.T. (1977), Regulation of nitrogen fixation in Rhizobium spp. Isolation of mutants of Rhizobium trifolii which induce nitrogenase activity. Biochimica et Biophysica Acta. 500. 277-290.

O'GARA, F. and SHANMUGAM, K.T. (1978), Mutant strains of clover rhizobium (Rhizobium trifolii) that form nodules on soybean (Glycine max). Proceedings of the National Academy of Sciences of the United States of America. 75. 2343-2347.

ORME-JOHNSON, W.H. (1977), Biochemistry of nitrogenase, in 'Genetic engineering for nitrogen fixation'. Ed. A. Hollaender, pp 317-331, New York and London: Plenum Press.

ORME-JOHNSON, W.H., DAVIS, L.C., HENZL, M.T., AVERILL, B.A., ORME-JOHNSON, N.R., MUNCK, E. and ZIMMERMAN, R. (1977), Components and pathways in biological nitrogen fixation, in 'Recent developments in nitrogen fixation'. Ed. W. Newton, J.R. Postgate and C. Rodriguez-Barrueco, pp. 131-178, London: Academic Press.

PAAU, A.S., LEE, D. and COWLES, J.R. (1977), Comparison of nucleic acid content in populations of free-living and symbiotic Rhizobium meliloti by flow microfluorometry, Journal of Bacteriology, 129, 1156-1158.

PAGAN, J.D., CHILD, J.J., SCOWCROFT, W.R. and GIBSON, A.H. (1975), Nitrogen fixation by Rhizobium cultured on a defined medium, Nature, 256, 406-407.

PAGAN, J.D., SCOWCROFT, W.R., DUDMAN, W.F. and GIBSON, A.H. (1977), Nitrogen fixation in nitrate reductase-deficient mutants of cultured rhizobia, Journal of Bacteriology, 129, 718-723.

PAHEL, G., ZELENTZ, A.D. and TYLER, B.M. (1978), glnB gene and regulation of nitrogen metabolism by glutamine synthetase in Escherichia coli, Journal of Bacteriology, 133, 139-148.

PANKHURST, C.E. (1974), Ineffective Rhizobium trifolii mutants examined by immune-diffusion, gel-electrophoresis and electron microscopy, Journal of General Microbiology, 82, 405-413.

PANKHURST, C.E. (1977), Symbiotic effectiveness of antibiotic-resistant mutants of fast- and slow-growing strains of Rhizobium nodulating Lotus species, Canadian Journal of Microbiology, 23, 1026-1033.

PANKHURST, C.E. and CRAIG, A.S. (1978), Effect of oxygen concentration, temperature and combined nitrogen on the morphology and nitrogenase activity of Rhizobium sp. strain 32HI in agar culture, Journal of General Microbiology, 106, 207-219.

PANKHURST, C.E., SCHWINGHAMER, E.A. and BERGERSEN, F.J. (1972), The structure and acetylene-reducing activity of root nodules formed by a riboflavin-requiring mutant of Rhizobium trifolii. Journal of General Microbiology, 70, 161-177.

PANKHURST, C.E., SCHWINGHAMER, E.A., THORNE, S.W. and BERGERSEN, F.J. (1974), The flavin content of clovers relative to symbiosis with a riboflavin-requiring mutant of Rhizobium trifolii. Plant Physiology, 53, 198-205.

PASTAN, I. and PERLMAN, R.L. (1969), Repression of β-galactosidase synthesis by glucose in phosphotransferase mutants of Escherichia coli. Journal of Biological Chemistry, 244, 5836-5842.

PASTAN, I. and PERLMAN, R.L. (1970), Cyclic adenosine monophosphate in bacteria, Science, 169, 339-344.

PEDROSA, F.O. and ZANCAN, G.T. (1974), L-arabinose metabolism in Rhizobium japonicum, Journal of Bacteriology, 119, 336-338.

PERLMAN, R.L. and PASTAN I. (1969), Pleiotropic deficiency of carbohydrate utilization in an adenyl cyclase deficient mutant of Escherichia coli. Biochemical and Biophysical Research Communications, 37, 151-157.

PETERKOFSKY, A. (1977), Regulation of Escherichia coli adenylate cyclase by phosphorylation-dephosphorylation, Trends in Biochemical Sciences, 2, 12-14.

PHIBBS, P.V., FEARY, T.W. and BLEVINS, W.T. (1974), Pyruvate carboxylase deficiency in pleiotropic carbohydrate-negative mutant strains of Pseudomonas aeruginosa, Journal of Bacteriology, 118, 999-1009.

PHIBBS, P.V., McCOWEN, S.M., FEARY, T.W. and BLEVINS, W.T. (1978), Mannitol and fructose catabolic pathways of Pseudomonas aeruginosa. Carbohydrate-negative mutants and pleiotropic effects of certain enzyme deficiencies, Journal of Bacteriology, 133, 717-728.

PHILLIPS, D.A. (1974), Factors affecting the reduction of acetylene by Rhizobium-soybean cell associations in vitro, Plant Physiology, 53, 67-72.

PHILLIPS, D.A., NEWELL, K.D., HASSELL, S.A. and FELLING, C.E. (1976), The effect of CO_2 enrichment on root nodule development and symbiotic N_2 reduction in Pisum sativum L, American Journal of Botany, 63, 356-362.

PLANQUE, K. and KIJNE, J.W. (1977), Binding of pea lectins to a glycan type polysaccharide in the cell walls of Rhizobium leguminosarum, FEBS Letters, 73, 64-66.

PLANQUE, K., KENNEDY, I.R., DE VRIES, G., QUISPEL, A. and van BRUSSEL, A.A.N. (1977), Location of nitrogenase and ammonia-assimilatory enzymes in bacteroids of Rhizobium leguminosarum and Rhizobium lupini, Journal of General Microbiology, 102, 95-104.

PLANQUE, K., DE VRIES, G.E. and KIJNE, J.W. (1978), The relationship between nitrogenase and glutamine synthetase in bacteroids of Rhizobium leguminosarum of various ages, Journal of General Microbiology, 106, 173-178.

POSTGATE, J.R. (1972), The acetylene reduction test for nitrogen fixation, Methods in Microbiology, 6B, 343-356.

POSTMA, P.W. and ROSEMAN, S. (1976), The bacterial phosphoenolpyruvate: sugar phosphotransferase system, Biochimica et Biophysica Acta, 457, 213-257.

POTTS, J.R. and CLARKE, P.H. (1976), The effect of nitrogen limitation on catabolite repression of amidase, histidase and urocanase in Pseudomonas aeruginosa, Journal of General Microbiology, 93, 377-387.

PRIVAL, M.J., BRENCHLEY, J.E. and MAGASANIK, B. (1973), Glutamine synthetase and the regulation of histidase formation in Klebsiella aerogenes, Journal of Biological Chemistry, 248, 4334-4344.

PRIVAL, M.J. and MAGASANIK, B. (1971), Resistance to catabolite repression of histidase and proline oxidase during nitrogen limited growth of Klebsiella aerogenes, Journal of Biological Chemistry, 246, 6288-6296.

QUISPEL, A. (ed.)(1974). 'The Biology of Nitrogen Fixation'. Amsterdam and Oxford: North-Holland.

RAO, V.R., DARROW, R.A. and KEISTER, D.L. (1978), Effect of oxygen tension on nitrogenase and on glutamine synthetases I and II in Rhizobium japonicum 61A76. Biochemical and Biophysical Research Communications, 81, 224-231.

REIJNDERS, L., VISER, L., AALBERS, A.M.J., van KAMMEN, A. and HOUWERS, A. (1975), A comparison of DNA from free living and endosymbiotic Rhizobium leguminosarum (strain PRE), Biochimica et Biophysica Acta, 414, 206-216.

RIGAUD, J., BERGERSEN, F.J., TURNER, G.L. and DANIEL, R.M. (1973), Nitrate dependent anaerobic acetylene-reduction and nitrogen fixation by soybean bacteroids, Journal of General Microbiology, 77, 137-144.

ROBERTSON, J.G. and TAYLOR, M.P. (1973), Acid and alkaline invertases in roots and nodules of Lupinus angustifolius infected with Rhizobium lupini, Planta, 112, 1-6.

ROBERTSON, J.G., FARNDEN, K.J.F., WARBURTON, M.P. and BANKS, J.M. (1975a), Induction of glutamine synthetase during nodule development in lupins, Australian Journal of Plant Physiology, 2, 265-272.

ROBERTSON, J.G., WARBURTON, M.P. and FARNDEN, K.J.F. (1975b), Induction of glutamate synthase during nodule development in lupin, FEBS Letters, 55, 33-37.

ROBERTSON, J.G., LYTTLETON, P., BULLIVANT, S. and GRAYSTON, G.F. (1978a), Membranes in lupin root nodules, I. The role of Golgi bodies in the biogenesis of infection threads and peribacteroid membranes, Journal of Cell Science, 30, 129-149.

ROBERTSON, J.G., WARBURTON, M.P., LYTTLETON, P., FORDYCE A.M. and BULLIVANT, S. (1978b), Membranes in lupin root nodules, II. Preparation and properties of peribacteroid membranes and bacteroid envelope inner membranes from developing lupin nodules, Journal of Cell Science, 30, 151-174.

ROMANO, A.H., EBERHARD, S.J., DINGLE, S.L. and McDOWELL, S.D. (1970), Distribution of the phosphoenolpyruvate: glucose phosphotransferase system in bacteria, Journal of Bacteriology, 104, 803-813.

ROWELL, P. and STEWART, W.D.P. (1976), Alanine dehydrogenase of the N_2-fixing blue-green alga, Anabaena cylindrica, Archives for Microbiology, 107, 115-124.

RUSSELL, P.E. and JONES, D.G. (1973), The effect of ultraviolet light on the symbiotic effectiveness and some physiological characteristics of four strains of Rhizobium trifolii, Journal of Applied Bacteriology, 36, 567-573.

RUTTER, W.J., HUNSLEY, J.R., GROVES, W.E., CALDER, J., RAJKUMAR, T.V. and WOODFIN, B.M. (1966), Fructose diphosphate aldolase, Methods in Enzymology, 9, 479-498.

RYAN, E. and FOTTRELL, P.F. (1974), Subcellular localization of enzymes involved in the assimilation of ammonia by soybean root nodules, Phytochemistry, 13, 2647-2652.

SADOFF, H.L. (1966), Glucose dehydrogenases-soluble. I. Bacillus cereus, Methods in Enzymology, 9, 103-107.

SAIER, M.H. (1977), Bacterial phosphoenolpyruvate: sugar phosphotransferase systems: structural,functional,and evolutionary inter-relationships, Bacteriological Reviews, 41, 856-871.

SAIER, M.H. and FEUCHT, B.U. (1975), Co-ordinate regulation of adenylate cyclase and carbohydrate permeases by the phosphoenolpyruvate: sugar phosphotransferase system in Salmonella typhimurium, Journal of Biological Chemistry, 250, 7078-7080.

SAIER, M.H. and MOCZYDLOWSKI, E.G. (1978), The regulation of carbohydrate transport in Escherichia coli and Salmonella typhimurium, in 'Bacterial transport'. Ed. B.P. Rosen, pp 103-125, Basel: Marcel Dekker.

SAIER, M.H. and ROSEMAN, S. (1976a), Sugar Transport. Inducer exclusion and regulation of the melibiose,maltose, glycerol and lactose transport systems by the phosphoenolpyruvate:sugar phosphotransferase system, Journal of Biological Chemistry, 251, 6606-6615.

SAIER, M.H. and ROSEMAN, S. (1976b), Sugar transport. The crr mutation: its effect on repression of enzyme synthesis, Journal of Biological Chemistry, 251, 6598-6605

SAIER, M.H. and SIMONI, R.D. (1976), Regulation of carbohydrate uptake in gram-positive bacteria, Journal of Biological Chemistry, 251, 893-894.

SAIER, M.H., SIMONI, R.D. and ROSEMAN, S. (1976a), Sugar transport: properties of mutant bacteria defective in proteins of the phosphoenolpyruvate:sugar phosphotransferase system, Journal of Biological Chemistry, 251, 6584-6597.

SAIER, M.H., FEUCHT, B.U. and HOFSTADTER, L.J. (1976b), Regulation of carbohydrate uptake and adenylate cyclase activity mediated by the enzymes II of the phosphoenolpyruvate:sugar phosphotransferase system in Escherichia coli, Journal of Biological Chemistry, 251, 883-892.

SAIER, M.H., STRAUD, H., MASSMAN, L.S., JUDICE, J.J., NEWMAN, M.J. and FEUCHT, B.U. (1978), Permease-specific mutations in Salmonella typhimurium and Escherichia coli that release the glycerol, maltose, melibiose and lactose transport sytems from regulation by the phosphenolpyruvate:sugar phosphotransferase system, Journal of Bacteriology, 133, 1358-1367.

St.JOHN, R.T., SHAH, V.K. and BRILL, W.J. (1974), Regulation of nitrogenase synthesis by oxygen in Klebsiella pneumoniae, Journal of Bacteriology, 119, 266-269.

SANDERS, R.E., CARLSON, R.W. and ALBERSHEIM, P. (1978), A Rhizobium mutant incapable of nodulation and normal polysaccharide secretion, Nature, 271, 240-242.

SAWYER, M.H., BAUMANN, P., BAUMANN, L., BERMAN, S.M., CANOVAS, J.L. and BERMAN, R.H. (1977), Pathways of D-fructose catabolism in species of Pseudomonas, Archives for Microbiology, 112, 49-55.

SCHINDLER, F. (1885), Ueber die biologische Bedeutung der Wurzelknöllchen bei der Papilionaceen, Journal für Landwirthschaft, 33, 325-336.

SCHUBERT, K.R. and EVANS, H.J. (1976), Hydrogen evolution: a major factor affecting the efficiency of nitrogen fixation in nodulated symbionts, Proceedings of the National Academy of Sciences of the United States of America, 73, 1207-1211.

SCHUBERT, K.R., ENGELKE, J.A., RUSSELL, S.A. and EVANS, H.J. (1977), Hydrogen reactions of nodulated leguminous plants, I. Effect of rhizobial strain and plant age, Plant Physiology, 60, 651-654.

SCHWINGHAMER, E.A. (1960), Studies on induced variation in the rhizobia. I. Defined media and nodulation test techniques, Journal of Applied Microbiology, 8, 349-352.

SCHWINGHAMER, E.A. (1964), Association between antibiotic resistance and ineffectiveness in mutant strains of Rhizobium spp, Canadian Journal of Microbiology, 10, 221-233.

SCHWINGHAMER, E.A. (1967), Effectiveness of Rhizobium as modified by mutation for resistance to antibiotics, Antonie van Leeuwenhoek, Journal of Microbiology and Serology, 33, 121-136.

SCHWINGHAMER, E.A. (1968), Loss of effectiveness and infectivity in mutants of Rhizobium resistant to metabolic inhibitors, Canadian Journal of Microbiology, 14, 355-367.

SCHWINGHAMER, E.A. (1970), Requirement for riboflavin for effective symbiosis on clover by an auxotrophic mutant strain of Rhizobium trifolii, Australian Journal of Biological Sciences, 23, 1187-1196.

SCHWINGHAMER, E.A. (1977), Genetic aspects of nodulation and dinitrogen fixation by legumes: the microsymbiont, in 'A treatise on dinitrogen fixation, Section III: Biology'. Ed. R.W.F. Hardy and W.S. Silver, pp 577-622, New York and London, Wiley-Interscience.

SCOTT, D.B., FARNDEN, K.J.F. and ROBERTSON, J.G. (1976), Ammonia assimilation in lupin nodules, Nature, 263, 703-705.

SCOWCROFT, W.R. and GIBSON, A.H. (1975), Nitrogen fixation by Rhizobium associated with tobacco and cowpea cell cultures, Nature, 253, 351-352.

SENIOR, P.J. (1975), Regulation of nitrogen metabolism in Escherichia coli and Klebsiella aerogenes: studies with the continuous-culture technique, Journal of Bacteriology, 123, 407-418.

SHAH, V.K. and BRILL, W.J. (1977), Isolation of an iron-molybdenum co-factor from nitrogenase, Proceedings of the National Academy of Sciences of the United States of America, 74, 3248-3253.

SHANMUGAM, K.T., CHAN, I. and MORANDI, C. (1975), Regulation of nitrogen fixation. Nitrogenase-derepressed mutants of Klebsiella pneumoniae, Biochimica et Biophysica Acta, 408, 101-111.

SHANMUGAM, K.T. and VALENTINE, R.C. (1975), Molecular biology of nitrogen fixation, Science, 187, 919-924.

SHAPIRO, B.M. and STADTMAN, E.R. (1970), Glutamine synthetase (Escherichia coli), Methods in Enzymology, 17A, 910-922.

SIDLOI-LUMBROSO, R., KLEIMAN, L. and SCHULMAN, H.M. (1978) Biochemical evidence that leghaemoglobin genes are present in the soybean but not in the Rhizobium genome, Nature, 273, 558-560.

SIEGEL, L.S., HYLEMON, P.B. and PHIBBS, P.V. (1977), Cyclic adenosine 3', 5'-monophosphate levels and activities of adenylate cyclase and cyclic adenosine 3', 5'-monophosphate phosphodiesterase in Pseudomonas and Bacteroides, Journal of Bacteriology, 129, 87-96.

SIK, T. and BARABÁS, I. (1977), The correlation of nitrate reductase and nitrogenase in Rhizobium meliloti, in 'Recent developments in nitrogen fixation'. Ed. W. Newton, J.R. Postgate and C. Rodriguez-Barrueco, pp 365-373, London: Academic Press.

SILSBURY, J.H. (1977), Energy requirement for symbiotic nitrogen fixation, Nature, 267, 149-150.

SKINNER, K.J. (1976), Nitrogen fixation - Special Report, Chemical and Engineering News, 54, 22-35.

SKOTNICKI, M.L. and ROLFE, B.G. (1978), Transfer of nitrogen fixation genes from a bacterium with the characteristics of both Rhizobium and Agrobacterium, Journal of Bacteriology, 133, 518-526.

SMYTH, P.F. and CLARKE, P.H. (1975), Catabolite repression of Pseudomonas aeruginosa amidase. The effect of carbon source on amidase synthesis, Journal of General Microbiology, 90, 81-90.

SOLS, A. and SALAS, M.L. (1966), Phosphofructokinase, Methods in Enzymology, 9, 436-442.

SPRENT, J.I. (1976), Nitrogen fixation by legumes subjected to water and light stresses, in 'Symbiotic nitrogen fixation in plants', International Biological Programme, Vol 7. Ed. P.S. Nutman, pp 405-420, Cambridge: Cambridge University Press.

STOUTHAMER, A.H., de HAAN, P.G. and NIJKAMP, H.J.J. (1965), Mapping of purine markers in Escherichia coli K12, Genetical Research, Cambridge, 6, 442-453.

STREETER, J.G. (1974), Growth of two soybean shoots on a single root, Journal of Experimental Botany, 25, 189-198.

STREETER, J.G. and BOSLER, M.E. (1976), Carbohydrates in soybean nodules: identification of compounds and possible relationships to nitrogen fixation, Plant Science Letters, 7, 321-329.

STREICHER, S.L., SHANMUGAM, K.T., AUSUBEL, F., MORANDI, C. and GOLDBERG, R.B. (1974), Regulation of nitrogen fixation in Klebsiella pneumoniae: evidence for a role of glutamine synthetase as a regulator of nitrogenase synthesis, Journal of Bacteriology, 120, 815-821.

SUTTON, W.D. (1974), Some features of the DNA of Rhizobium bacteroids and bacteria, Biochimica et Biophysica Acta, 366, 1-10.

SUTTON, W.D. and MAHONEY, P. (1977), Preparation and fractionation of Rhizobium bacteroids by zone sedimentation through sucrose gradients, Plant Physiology, 60, 800-802.

SUTTON, W.D. and ROBERTSON, J.G. (1974), Control of gene action in nitrogen fixing bacteroids, in 'Mechanisms of regulation of plant growth'. Ed. R.L. Bieleski, A.R. Ferguson and M.M. Cresswell, pp 23-30, Wellington: The Royal Society of New Zealand.

SUTTON, W.D., JEPSEN, N.M. and SHAW, B.D. (1977), Changes in the number, viability and amino-acid incorporating activity of Rhizobium bacteroids during lupin nodule development, Plant Physiology, 59, 741-744.

SVÁB, Z., OROSZ, L., KONDOROSI, Á. and SIK, T. (1973), Study of a specially transducing Rhizobium phage, Acta Microbiologica Academiae Scientarium Hungaricae, 20, 24.

SVÁB, Z., KONDOROSI, Á. and OROSZ, L. (1978), Specialized transduction of a cysteine marker by Rhizobium meliloti phage 16-3, Journal of General Microbiology, 106, 321-327.

SYONO, K., NEWCOMB, W. and TORREY, J.G. (1976), Cytokinin production in relation to the development of pea root nodules, Canadian Journal of Botany, 18, 2155-2162.

TEMPEST, D.W., MEERS, J.L. and BROWN, C.M. (1973), Glutamate synthetase (GOGAT): a key enzyme in the assimilation of ammonia by prokaryotic organisms, in 'The enzymes of glutamine metabolism', Ed. S. Prusiner and E.R. Stadtman, pp 167-182, New York: Academic Press.

TJEPKEMA, J.D. and EVANS, H.J. (1975), Nitrogen fixation by free-living Rhizobium in defined liquid medium, Biochemical and Biophysical Research Communications, 65, 625-628.

TRINICK, M.J. (1973), Symbiosis between Rhizobium and the non-legume, Trema aspera, Nature, 244, 459-460.

TRUCHET, G. (1972), Mise en évidence de l'activité peroxydasique dans les différentes zones des nodules radiculaires de pois (Pisum sativum L): localisation de la leghémoglobine, Comptes rendus hebdomadaire des seances de l'Academie des Sciences, Paris, 274, 1290-1293.

TSEIN, H.C., CAIN, P.S. and SCHMIDT, E.L. (1977), Viability of Rhizobium bacteroids, Applied and Environmental Microbiology, 34, 854-856.

TSHITENGE, G., LUYINDULA, N., LURQUIN, P.F. and LEDOUX, L. (1975), Plasmid deoxyribonucleic acid in Rhizobium vigna and Rhizobium trifolii, Biochimica et Biophysica Acta, 414, 357-361.

TU, J.C. (1975), Structural similarity of the membrane envelopes of rhizobial bacteroids and the host plasma membrane as revealed by freeze-fracturing, Journal of Bacteriology, 122, 691-694.

TUBB, R.S. (1974), Glutamine synthetase and ammonium regulation of nitrogenase synthesis in Klebsiella, Nature, 251, 481-485.

TUBB, R.S. (1976), Regulation of nitrogen fixation in Rhizobium sp., Applied and Environmental Microbiology, 32, 483-488.

TUBB, R.S. and POSTGATE, J.R. (1973), Control of nitrogenase synthesis in Klebsiella pneumoniae, Journal of General Microbiology, 79, 103-117.

TUZIMURA, K. and MEGURO, H. (1960), Respiration substrate of Rhizobium in the nodules, Journal of Biochemistry, 47, 391-397.

TYLER, B., DE LEO, A.B. and MAGASANIK, B. (1974), Activation of transcription of hut DNA by glutamine synthetase, Proceedings of the National Academy of Sciences of the United States of America, 71, 225-229

ULLMANN, A. (1974), Are cyclic AMP effects related to real physiological phenomena? Biochemical and Biophysical Research Communications, 57, 348-352.

UPCHURCH, R.G. and ELKAN, G.H. (1978), The role of ammonia, L-glutamate and cyclic adenosine 3', 5'-monophosphate in the regulation of ammonia assimilation in Rhizobium japonicum, Biochimica et Biophysica Acta, 538, 244-248.

UTTER, M.F. and SCRUTTON, M.C. (1969), Pyruvate carboxylase, Current Topics in Cellular Regulation, 1, 253-296.

VERMA, D.P.S. and BAL, A.K. (1976), Intracellular site of synthesis and localization of leghaemoglobin in root nodules, Proceedings of the National Academy of Sciences of the United States of America, 73, 3843-3847.

VICENTE, M. and CÁNOVAS, J.L. (1973), Glucolysis in Pseudomonas putida: physiological role of alternative routes from the analysis of defective mutants, Journal of Bacteriology, 116, 908-914.

VINCENT, J.M. (1970), 'A Manual for the Practical Study of Root-Nodule Bacteria. International Biological Programme Handbook, No. 15.' Oxford: Blackwell Scientific Publications.

VINCENT, J.M. (1977), Rhizobium: general microbiology, in 'A treatise on dinitrogen fixation, Section III: Biology'. Ed. R.W.F. Hardy and W.S. Silver, pp 277-366, New York: Wiley Interscience.

VIRTANEN, A.I., ERKAMA, J. and LINKOLA, H. (1947), On the relation between nitrogen fixation and leghaemoglobin content of leguminous root nodules, II. Acta Chemica, Scandinavia, 1, 861-870.

WALKER, M.N. and MORTENSON, L.E. (1974), Evidence for the existence of a fully reduced state of molybdoferredoxin during the functioning of nitrogenase, and the order of electron transfer from reduced ferredoxin, Journal of Biological Chemistry, 249, 6356-6358.

WHITING, M.J. and DILWORTH, M.J. (1974), Legume root nodule nitrogenase: purification, properties and studies on its genetic control, Biochimica et Biophysica Acta, 371, 337-351.

WHITING, P.H., MIDGLEY, M. and DAWES, E.A. (1976a), The regulation of transport of glucose, gluconate and 2-oxogluconate and of glucose catabolism in Pseudomonas aeruginosa, Biochemical Journal, 154, 659-668.

WHITING, P.H., MIDGLEY, M. and DAWES, E.A. (1976b), The role of glucose limitation in the regulation of the transport of glucose, gluconate and 2-oxogluconate, and of glucose metabolism in Pseudomonas aeruginosa, Journal of General Microbiology, 92, 304-310.

WITTENBERG, J.B., BERGERSEN, F.J., APPLEBY, C.A. and TURNER, G.L. (1974), Facilitated oxygen diffusion. The role of leghaemoglobin in nitrogen fixation by bacteroids isolated from soybean root nodules, Journal of Biological Chemistry, 249, 4056-4066.

WOHLHEUTER, R.M., SCHUTT, H. and HOLZER, H. (1973), Regulation of glutamine synthetase in vivo in E. coli, in 'The enzymes of glutamine metabolism'. Ed. S. Prusiner and E.R. Stadtman, pp 45-64, New York: Academic Press.

WOLPERT, J.S. and ALBERSHEIM, P. (1976), Host-symbiont interactions, I. The lectins of legumes interact with the O-antigen-containing lipopolysaccharides of their symbiont rhizobia, Biochemical and Biophysical Research Communications, 70, 729-737.

WONG, P.P. and EVANS, H.J.(1971), Poly-β-hydroxybutyrate utilization by soybean (Glycine max Merr.) nodules and assessment of its role in maintenance of nitrogenase activity, Plant Physiology, 47, 750-755.

WONG, P.P., EVANS, H.J., KLUCAS, R.V. and RUSSEL, S. (1971), Investigations into the pathway of electron transport to the nitrogenase from nodule bacteroids, Plant and Soil, Special Volume, 525-543.

WU, T.T. (1976), Growth on D-arabitol of a mutant strain of Escherichia coli K12 using a novel dehydrogenase and enzymes related to L-1,2-propanediol and D-xylose metabolism, Journal of General Microbiology, 94, 246-256.

YATES, M.G. (1977), Physiological aspects of nitrogen fixation, in 'Recent developments in nitrogen fixation'. Ed. W. Newton, J.R. Postgate and C. Rodriguez-Barrueco, pp 219-270, London: Academic Press.

YATES, M.G. and JONES, C.W. (1974), Respiration and nitrogen fixation in Azotobacter, Advances in Microbial Physiology, 11, 97-135.

YATES, M.G., THORNELEY, R.N.F. and LOWE, D.J. (1975), Nitrogenase of Azotobacter chroococcum: inhibition by ADP of the reduction of oxidised Fe protein by sodium dithionite, FEBS Letters, 60, 89-93.

YOCH, D.C. (1974), Electron transport carriers involved in nitrogen fixation by the coliform Klebsiella pneumoniae, Journal of General Microbiology, 83, 153-164.

YOUNG, M.R., TOLBERT, B. and UTTER, M.F. (1969), Pyruvate carboxylase from Saccharomyces cerevisiae, Methods in Enzymology, 13, 250-258.

ZIMMERMANN, F.K. and SCHEEL, I. (1977), Mutants of Saccharomyces cerevisiae resistant to carbon catabolite repression, Molecular and General Genetics, 154, 75-82.

ZUMFT, W.G., MORTENSON, L.E. and PALMER, G. (1974), Electron-paramagnetic-resonance studies on nitrogenase. Investigation of the oxidation-reduction behaviour of azoferredoxin and molybdoferredoxin with potentiometric and rapid-freeze techniques, European Journal of Biochemistry, 46, 525-535.

ZUMFT, W.G. and MORTENSON, L.E. (1975), The nitrogen-fixing complex of bacteria, Biochimica et Biophysica Acta, 416, 1-52.

ZURKOWSKI, W., HOFFMAN, M. and LORKIEWICZ, Z. (1973), Effect of acriflavine and sodium dodecyl sulphate on infectiveness of Rhizobium trifolii, Acta Microbiologica Polonica, Ser.A, 5, 55-60.

ZURKOWSKI, W. and LORKIEWICZ, Z. (1976), Plasmid deoxyribonucleic acid in Rhizobium trifolii, Journal of Bacteriology, 128, 481-484.

BIBLIOGRAPHY

1. RONSON, C.W. and PRIMROSE, S.B. (1977).
Regulatory aspects of carbohydrate metabolism in Rhizobium trifolii
Proceedings of the Society for General Microbiology, 4, 147-148.

2. RONSON, C.W. and PRIMROSE, S.B. (1979)
Carbohydrate metabolism in Rhizobium trifolii: identification and symbiotic properties of mutants.
Journal of General Microbiology (in press).

APPENDIX: LIST OF *R. trifolii* MUTANT STRAINS USED

Strain	Parent	Relevant characteristics
7000		wild-type
7008	7000	*trp*-4
7009	7008	*trp*-4 *glk*-4
7010	7009	*trp*-4 *glk*-4 Dul$^+$
7011	7010	*trp*-4 *glk*-4 Dul$^+$ (Dul + 2DG)$^+$
7012	7000	*his*-6
7013	7012	*his*-6 *glk*-7
7014	7009	*glk*-4
7015	7012	*his*-6 *glk*-1 Sam$^-$
7017	7012	*his*-6 *sam*-7
7021	7013	*his*-6 Glc$^+$ Sam$^-$
7027	7015	*his*-6 Glc$^+$ Sam$^+$
7028	7012	*his*-6 *edp*-1
7029	7009	*trp*-4 *glk*-4 Lac$^+$
7039	7013	*his*-6 *glk*-7 *fup*-1
7046	7015	*his*-6 *glk*-1 Glc$^-$ Sam$^+$
7049	7000	*pyc*-2
7056	7000	*pyc*-18
7062	7008	*trp*-4 *glk*-2
7083	7026	*his*-6 Glc$^+$ Sam$^-$ Gal$^-$
7084	7000	*nal*-1
7085	7000	*nal*-2
7092	7000	*pur*-2 *rif*-2 *str*-2
7093	7000	*pur*-3 *rif*-3 *str*-3
7094	7012	*his*-6 *ura*-4
7095	7008	*trp*-4 *pur*-4 *str*-4
7097	7008	*trp*-4 *pur*-4 *rif*-4
W 19		wild-type
W 1903	W 19	*pur*-1 *rif*-1 *str*-1